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Identification and functional characterization of epigenetic determinants of pancreatic CSCs

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This thesis, submitted for the degree of Doctor of Philosophy
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under the supervision of Prof. Dr. Christopher Heeschen.

Dedicated to my parents,
my sister Ivana and my
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Bruno, I was thinking when I come to the part to thank you that words will just start to flow. Instead I am stunned how small words are. When you took me under your ''scientific shelter'', I knew that doesn't matter how hard science is, and how difficult is to keep up being motivated, that having someone brilliant next to, is sufficient to overcome all obstacles. Hours you spent with me, lessons you thought me, times when you had a handkerchief for me…all these things you have done for me, since the begging and at the end. Was an honour growing up as a scientist next to you. Hope that one day I would be able to pay you back. Thank you for everything!!!

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At the end I think I will keep on pipetting and thanks to people I mentioned I hope I have set up good grounds to one day make a difference.

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SUMMARY

Human tumours including pancreatic cancer display significant functional heterogeneity in their respective cancer cell populations. Cancer stem cells (CSCs) represent a subpopulation of cells distinguishable from the bulk of the tumour by their exclusive ability to drive tumourigenesis based on their unlimited self-renewal capacity and their ability to give rise to more differentiated progenies (non-CSCs). Since CSCs and non-CSCs share an identical genetic background, the purpose of our study is to identify and functionally characterize the epigenetic signature of pancreatic CSCs in order to better understand the differences between these highly tumourigenic cells and their less tumourigenic progenies. Using high-throughput methylation analysis, we discovered that CSCs (i.e autofluorescent-positive cells) have a higher level of global methylation compared to their negative counterparts, regardless of the heterogeneity or polyclonality of the CSC populations present in the tumours analysed. Moreover, we found that CSCs express higher level of DNMT methylentrasferase proteins, with DNMT1 showing the most consistent up-regulation, which makes this protein a potentially attractive target for chemotherapy and chemoprevention. Specifically, targeting DNMT1 in CSCs using the DNMT inhibitor Zebularine significantly reduced their sphere formation capacity and *in vivo* tumourigenic potential. This inhibitory effect on CSCs, is mediated, in part, through epigenetic regulation of microRNAs, specifically, the cluster miR-17-92. Furthermore, we discovered that CSCs and non-CSCs have different expression level of proteins involved in DNA demethylation, with the TET2 protein being expressed at low levels in CSCs. Subsequent loss-of-function experiments in more differentiated cells resulted in their increased self-renewal, higher migration and *in vivo* tumourigenicity. Together our findings indicate that DNA methylation and demethylation play a crucial role in CSC biology and at the same time highlight the realistic potential for developing modulators that could modulate the epigenetic plasticity of CSCs offering new therapies to improve the poor outcome of patients with pancreatic ductal adenocarcinoma.

Resumen

Los tumores humanos, incluyendo el adenocarcinoma ductal de páncreas (PDAC, del inglés pancreatic ductal adenocarcinoma), son muy heterogéneos respecto a las poblaciones de células cancerosas que contienen. Las células madre tumorales (CSCs, del inglés cancer stem cells) forman una subpoblación de células cancerosas caracterizada por su alta capacidad tumorigénica, autorenovación y su habilidad para repoblar el tumor. Dado que las CSCs y las células no-CSCs comparten el mismo perfil genético, el propósito de este estudio es identificar y caracterizar la firma epigenética de las CSCs pancreáticas que pueda explicar las diferencias funcionales entre estas células altamente tumorigénicas y sus progenies. Usando un análisis de metilación de alto rendimiento, hemos descubierto que las CSCs (identificadas como células autofluorescentes) tienen un nivel de metilación global más alto que las células diferenciadas, sin que influya el grado de heterogeneidad de los tumores analizados. Además, hemos encontrado que las CSCs expresan un mayor nivel de las metiltransferasas DNMT, sobre todo DNMT1, lo que convierte estas proteínas en una diana especialmente interesante para quimioterapia y quimioprevención. Específicamente, la inhibición de DNMT1 con zebularine redujo significativamente la capacidad de formar esferas y su potencial tumorigénico *in vivo*. Este efecto está parcialmente mediado por la regulación epigenética de los microRNAs de la familia miR-17-92. Además, hemos descubierto que las CSCs y las células tumorales diferenciadas expresan diferentes niveles de proteínas encargadas de demetilar el ADN, siendo la proteína TET2 la menos expresada en CSCs. Los experimentos de pérdida de función en células diferenciadas resultaron en una mayor capacidad de autorenovación, migración y tumorigénesis *in vivo*. En resumen, nuestros resultados indican que la metilación-demetilación del ADN tiene un papel crucial en la biología de las CSCs. Este hecho pone de relevancia que el desarrollo de moduladores de la plasticidad epigenética de las CSCs podría utilizarse como quimioterapia, potencialmente suponiendo una importante mejora de la situación de los pacientes con adenocarcinoma de páncreas.

ABBREVIATIONS

AML	Acute myeloid leukaemia
5-aza-CR	5-azacitydine
5-aza-CdR	5-aza-2-deoxycitydine
BSA	Bovine Serum Albumin
bFGF	Basic fibroblast growth factor
CK19	Cytokeratin 19
CDNK2A	Cyclin-dependent kinase inhibitor 2A
CML	Chronic myeloid leukaemia
CSC	Cancer Stem Cell
CSCs	Cancer Stem Cells
DAPI	4',6-diamidino-2-phenylindole
DFS	Disease free survival
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
DNMT	Deoxyribonucleic acid methyltranspherase
DNMT1	Deoxyribonucleic acid methyltranspherase 1
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cells
EpCAM	Epithelial cell adhesion molecule
5-FU	5-Fluoracil
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FFPE	Formalin-Fixed, Paraffin-Embedded
GBM	Glioblastoma
5-hmC	5-hydroxymethylcytosine
HRP	Horseradish peroxidase
HNU mice	Athymic Nude-Foxn1nu
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
mRNA	Messenger ribonucleic acid
miRNA	Micro- ribonucleic acid
NSCLC	Non-small cell lung cancer
OS	Overall survival
PDAC	Pancreatic ductal adenocarcinoma
PanINs	Pancreatic Intraepithelial Neoplasias
PaCSCs	Pancretic cancer stem cells
PSCs	Pancretic stellate cells
PBS	Phosphate buffered saline
PEN	Penicillin
RT-qPCR	real time quantitive polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
STREP	Streptomycin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMAD4	SMAD Family Member 4
TET2	Tet Methylcytosine Dioxygenase 2
TBS	Tris-buffered saline
TSG	Tumour suppressor gene
TGF-ß1	Transforming growth factor beta 1

INTRODUCTION

1. PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC), the most frequent form of pancreatic cancer, is a common malignancy, with around 280,000 new cases being diagnosed worldwide in 2008, and 70,000 cases seen in the European Union alone (Hassan et al., 2007; Hidalgo et al., 2015). The overall 5-year survival rate of this disease is less than 5%, and it is expected that by the 2020, pancreatic ductal adenocarcinoma (PDAC) will be second only to non-small-cell lung cancer (NSCLC) as the leading cause of cancer-related mortality in the USA (Garrido-Laguna and Hidalgo, 2015). These alarming statistics are primarily due to the fact that there are no specific symptoms that can act as accurate predictive indicators of early stage pancreatic cancer; consequently, most patients present with advanced-stage disease in which the cancer has already metastasized to distant secondary organs (Greer and Brand, 2007). Thus far, no serum or tumour-based biomarkers or biomarker panels have been established that are both sensitive and specific enough for accurate early detection purposes. Very recently a study published by researchers at The University of Texas MD Anderson Cancer Centre showed that the protein encoded by the gene glypican-1 (GPC1) is present on cancer exosomes and may be used as part of a potential non-invasive diagnostic and screening tool to detect early pancreatic cancer, potentially at a stage acceptable for pancreatico-duodenectomy (Melo et al., 2015), which can be curative if tumours are detected early. Unfortunately, the majority of tumours are not detected early enough and thus the 5-year survival rate for patients undergoing tumour resection still remains approximately 15-20% (Garrido-Laguna and Hidalgo, 2015). A new diagnostic based on glypican-1 has the potential to change this scenario. Nevertheless, there still remains an urgent need for more accurate and clear prognostic and predictive markers to better select patients for current therapies and for the development of new and novel therapeutic strategies for PDAC.

1.1. Risk factors for PDAC

Pancreatic cancer has a greater incidence in men than in women, increases with age, and predominates in certain ethnic/racial groups, such as African Americans, Ashkenazi Jews, Pacific Islanders, and New Zealand Maori (Greer and Brand, 2007). The increased risk among certain

populations appears to be multifactorial in nature and is likely to be due to a combination of environmental and inherited factors. Smoking has been identified as a clear risk factor since it has been demonstrated that smokers have a 3-times higher risk of developing pancreatic cancer compared to non-smokers (Hassan et al., 2007). In addition, diabetes and obesity appear to confer increased risk for the development of PDAC, and as many as 80% of pancreatic cancer patients have glucose intolerance or frank diabetes at the time of diagnosis (Berrington de Gonzalez et al., 2003). Chronic pancreatitis has also been shown to greatly increase the lifetime risk of pancreatic cancer development and this could be due in part to promoting the local release of cytokines and reactive oxygen species (ROS) that induce cell proliferation, disrupt cell differentiation states, and select for oncogenic mutations (Hezel et al., 2006).

1.2. Molecular biology of PDAC

Pancreatic ductal adenocarcinoma (PDAC) commonly arises in the head of the pancreas spreading into the surrounding tissue, such as lymph nodes, peritoneum and spleen (Hezel et al., 2006). PDAC frequently metastasizes to the liver and spleen; even the smallest primary lesions commonly exhibit lympho-vascular invasion, suggesting a propensity for early distant spread (Wolfgang et al., 2013). Like colon cancer, pancreatic cancer has been shown to undergo a stepwise progression from dysplasia to invasive adenocarcinoma. The precursor lesions can be divided into three groups based on clinical and histopathologic analyses: mucinous cystic neoplasms that harbour an ovarian-type stroma (MCN), intraductal papillary mucinous neoplasm that grow into larger cystic structures (IPMN) and the most studied, pancreatic intraepithelial neoplasias (PanINs) (Maitra et al., 2005). PanINs are classified into three stages of increasing cellular atypia and these epithelial changes are matched by desmoplastic changes in the stroma (Morris et al., 2010) (Figure I1). During PanIN-to-PDAC progression, several alterations occur, including: 1) telomere shortening that causes genetic instability (Mihaljevic et al., 2010), 2) *KRAS* mutations, which are the driving activating mutations acquired during early events (Avila et al., 2012) and occur in greater than 90% of PDAC patients, 3*) CDNK2A* inactivation resulting in P16 loss, which is a regulator of cell cycle G1-S transition (Lukas et al., 1995), 4) *P53* loss, although not as frequent as the other mutations (around 50-70%), its loss represents a deregulation of the cell cycle due to the lack of checkpoints for DNA damage control or apoptosis (Reinhardt et al.,

2007), 5) *SMAD4* mutations, present in ~50% of the cases. SMAD4 acts also as a tumour suppressor, and its loss or mutated form leads to the deregulation TGF-β signalling (Hidalgo and Von Hoff, 2012).

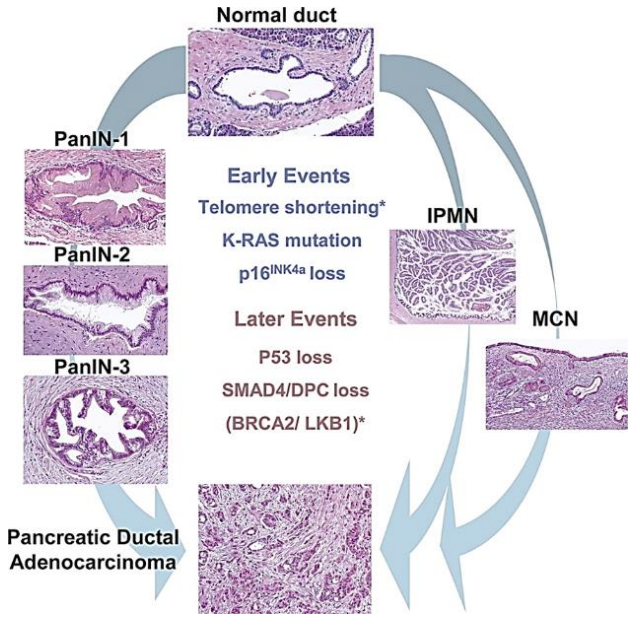

Figure I1. Representative illustration of the histopathological changes that occur during PDAC progression. There are three known human PDAC precursor lesions: PanIN, MCN, and IPMN. The PanIN grading scheme is shown on the left; increasing grade (1–3) reflects increasing atypia, eventually leading to adenocarcinoma. The right side illustrates the potential progression of MCNs and IPMNs to PDAC. *Adapted from (Hezel et al., 2006)*

1.3. Treatment options for PDAC patients

Pancreatic cancer is broadly classified into resectable, borderline resectable and advanced disease (Barugola et al., 2012). Unfortunately, in most cases, PDAC is already locally advanced at the time of diagnosis so that, as mentioned before, only 20% of cases are candidates for surgery and have CT evidence of no distant metastases (Tempero et al., 2012). The choice of surgical procedure depends on the location of the tumour: pancreatico-duodenectomy (Whipple procedure) for lesions of the head and neck; distal pancreatectomy for lesions of the body or tail; and total pancreatectomy in multifocal disease (Venkat et al., 2012). Chemotherapy, radiation therapy or a combination of both can be given prior to surgical resection (neoadjuvant therapy) with the aim of optimizing surgical resection or therapy can be given following surgical resection (adjuvant therapy) in order to consolidate surgery and improve cure rates. Although the benefit of

neoadjuvant and adjuvant therapy has improved in recent years, the best choice of treatment modality still remains highly controversial (Herreros-Villanueva et al., 2012).

The first randomized control trial (RCT) of adjuvant therapy in pancreatic cancer concluded that treatment with 5-fluorouracil (5-FU) plus radiation followed by two years of weekly 5-FU maintenance provided better outcomes than surgery alone (Kalser and Ellenberg, 1985). 5-FU is a thymidylate synthase inhibitor that blocks the synthesis of pyrimidine thymidine, a nucleotide required for DNA replication (Longley et al., 2003). This agent had been considered the only chemotherapeutic option for about 20 years until the introduction of gemcitabine in 1997. The development of gemcitabine was considered a major advance in the treatment of pancreatic cancer in the late 1990s (Rothenberg et al., 1996). This drug acts as a difluorinated analog of deoxycytidine and as a prodrug is phosphorylated by cytoplasmic and mitochondrial enzymes to its active metabolites-gemcitabine diphosphate and gemcitabine triphosphate. The cytotoxic effect of this drug is attributed to a combination of two actions of the diphosphate and triphosphate nucleosides, which lead to inhibition of DNA synthesis (Hertel et al., 1990). In operable disease, gemcitabine has been used in clinical trials as a single agent (Oettle et al., 2007) and showed significant improvement for median disease free survival (DFS) (gemcitabine (G):13.4 months (m), observation (O): 6.9m, p< .001). G significantly improved median OS (G: 22.8m, O: 20.2m, p=.005). Combined therapies are still under investigation and there is currently a phase III clinical trial comparing adjuvant gemcitabine versus gemcitabine and capecitabine (www.lctu.org.uk/trial/trial_info.asp?id=48&tgcode=4&menuid=30).

In metastatic disease, gemcitabine was originally found more effective than 5-FU in alleviation of some disease-related symptoms in patients with advanced, symptomatic disease, but only conferred a modest survival advantage over treatment with 5-FU. This pivotal phase III clinical trial demonstrated improvement in median overall and 1-year survival compared to 5-FU (5.7 mo vs 4.4 mo and 18% vs 2%, respectively) (Burris et al., 1997). As treatment with gemcitabine was associated with significant clinical response and better survival, this drug was approved as first line therapy of metastatic pancreatic cancer. Several drug combinations with gemcitabine have been tested since for treatment in metastatic disease. For example the epidermal growth factor receptor inhibitor, erlotinib, with gemcitabine versus gemcitabine alone showed marginally increased overall survival (OS) (6.24 v. 5.91 mo, p = 0.04) but greater toxicity in combination (Moore et al., 2007).

The important breakthrough in treatment of metastatic pancreatic cancer came with the introduction of Abraxane. Abraxane is an albumin bound paxitaxel that targets SPARK (also known as osteonectin and basement membrane-40 protein that is a member of the matricellular family of proteins that play crucial roles in tissue homeostasis (Chiodoni et al., 2010)). In preclinical studies, Abraxane, showed antitumour activity as a single agent and synergistic activity in combination with gemcitabine in murine models of pancreatic cancer (Frese et al., 2012). On the basis of preclinical evidence, a phase I-II clinical trial was conducted and showed promising efficacy, with a median survival of 12.2 months and a manageable safety profile. Subsequently, a phase III study showed that abraxane plus gemcitabine led to a significant improvement in survival at all-time points. In particular, the survival curves separated early, with a median improvement of 1.8 months and an improvement of 3.4 months at the time point when 25% of the patients were alive. The rate of survival was significantly higher in the abraxane– gemcitabine group than in the gemcitabine group alone - by 59% at 1 year (35% vs. 22%) and by more than 100% at 2 years (9% vs. 4%) (Von Hoff et al., 2013) . A pivotal trial in 2011 by Conroy *et al*. (Conroy et al., 2011) demonstrated a significant improvement in overall survival (OS) compared to previous gemcitabine containing-regimes. The combination of FOLFIRINOX (5-FU, leucovorin, irinotecan and oxaliplatin) showed a substantial increase in median overall survival over gemcitabine alone in metastatic pancreatic cancer, with an OS benefit of 11.1 months versus 6.8 months with gemcitabine alone. This trial importantly also demonstrated longer time to symptomatic deterioration for patients in the FORFIRINOX group. Again, however, higher-grade toxicity was seen with the combination treatment (Conroy et al., 2011). Thus this treatment regime is now considered the gold standard for patients with metastatic disease but who have a good performance status.

Although tremendous efforts have been invested in improving our therapeutic approaches for treating PDAC patients, virtually all patients inevitably succumb to the disease. Therefore, new approaches for targeting pancreatic cancer are still desperately needed to pave the way for the development of disease-free treatment regimens (Hermann et al., 2009; Mueller et al., 2009). Table I1 summarizes treatment options for patients with PDAC.

CLINICAL REGIMENS PROVEN TO INCREASE SURVIVAL FOR PATIENTS WITH ADVANCED METASTATIC PANCREATIC CANER				
Regimen	Control	Median Survival (months) Regimen	Control	Reference
Gemcitabine	5-FU	5.6	4.4	Burris, et al., 1997
Gemcitabine + Erlotinib	*GEM	6.24	5.91	Moore, et al., 2007
FOLFIRINOX**	*GEM	11.1	6.8	Conroy, et al., 2011
nab-paclitaxel + gemctibaine	*GEM	8.5	6.7	Von Hoff, et al., 2012
*GEM: gemcitabine **FOLFIRINOX: Folinic acid + 5 FU + Irinotecan + Oxaliplatin				

Table I1. Current treatments for PDAC cancer. *Adapted from (Von Hoff et al., 2013)*

2. CANCER STEM CELLS IN CANCER

2.1. The cancer stem cell concept in cancer

Despite immense research activity focused on developing more effective treatment options for patients with pancreatic cancer, the majority of PDAC patients fail to respond to standard of care resulting in disease progression, recurrence, and reduced overall survival (Goel and Sun, 2015). This could be due to the fact that the tumour is not simply a ‘’mass’’ of homogeneous tumour cells, but rather a complex ecosystem where apart from tumour cells, the tumour is made up of various infiltrating endothelial, hematopoietic, stromal cells such as pancreatic stellate cells (PSC) and immune cells (e.g. macrophages), all of which can influence the biology and overall status of the tumour as a whole. In line with this, cancer is now widely considered to be a heterogeneous disease and there is an increasing awareness that intratumoural heterogeneity contributes to therapy failure and disease progression (Hanahan and Weinberg, 2011).

Two models have been put forward to explain tumour heterogeneity. The stochastic model argues that tumours are biologically homogeneous. Functional heterogeneity in tumour cells would be due to random or stochastic influences that alter the behaviour of individual cells in the tumour (Dick, 2009). These influences can be extrinsic (for example microenvironment or immune response) or intrinsic (e.g. levels of transcription factors or specific signalling pathways). Multiple mutations occurring in a single cell can provide it with a selective growth advantage. As the tumour progresses, genetic instability and uncontrolled proliferation allow for the production of cells with

additional mutations and new characteristics. Through this process, any cancer cell can potentially become invasive and cause metastasis or become resistant to therapies and cause recurrence (Campbell and Polyak, 2007) (Figure I2A). This model, however, has some limitations. For example, the stochastic model predicts that every cell can initiate a tumour, which is in disagreement with tumourigenesis assays, where it has been observed that tens to thousands to millions of unfractionated cells have to be transplanted in order to transfer disease, suggesting that tumourigenic cells are very rare (Dick, 2008). Subsequent studies in which specific cell surface markers were used to isolate subpopulations of cells confirmed that tumourigenic potential was exclusive to a small enriched population of cells, and that the bulk tumour contained populations of cells with no tumourigenic capacity (Wang and Dick, 2005).

By contrast, the cancer stem cell hypothesis states that a particular subset of tumour cells with stem cell-like properties, called "cancer stem cells," drive tumour progression, and recurrence (Bonnet and Dick, 1997). By definition, these cells have the abilities to self-renew indefinitely and to differentiate. Their self-renewal and differentiation lead to the production of all cell types present within the tumour, thereby generating tumour heterogeneity (Reya et al., 2001). Meanwhile, the other cells in a tumour do not have unlimited self-renewal capacity and cannot differentiate to produce all tumour cell types (Figure I2B). In addition, according to the cancer stem cell hypothesis, tumour progression is a result of the metastatic spread of these cells, and cancer recurrence is caused by their resistance to therapy (Song and Miele, 2007). It cannot be ignored that the CSC model has some caveats, such as the use of specific cell surface markers to isolate CSCs, which may not adequately delineate the CSCs from non-CSCs. Moreover this model relies on xenotransplantation limiting dilution assays and assumes that the resulting increased tumourigenicity lies within an intrinsic CSC-specific trait to form tumours in immunocompromised mice, but the methodologies available to study CSCs cannot rule out that these cells may just somehow be better suited for growth in an immunocompromised mouse that bears little resemblance to the normal human environment. However, elegant lineage tracing experiments have clearly demonstrated that CSCs are a reservoir for tumourigenic capacity, are capable of surviving chemotherapy, and have ability to drive relapse in mouse models of cancer (Chen et al., 2012; Schepers et al., 2012). Also, recent studies have provided strong evidence supporting the clinical relevance of CSCs, such as in glioblastoma (GBM) where it was shown that a stem cell-like gene expression signature was associated with relapse and was predictive of patient

outcome in human leukemia, breast cancer, GBM and ovarian cancer (Balbous et al., 2014; Eppert et al., 2011). Of note, in leukemia, clinical studies have shown that in CML, chronic myelogenous leukemia, a cancer distinctly driven by stem cells, clonal evolution is observed upon treatment with tyrosine-kinase inhibitors (Wang et al., 2014). These observations highlight the fact that the progression of tumour heterogeneity is a very complex process and support the idea that clonal evolution and the CSC model are not mutually exclusive models (Figure I2C). In this scenario, a tumour may contain several cancer stem cell clones that are genetically and epigenetically distinct. Each different CSC subclone would give rise to intermediate progenitors as well as more differentiated, non-tumourigenic cancer cells. The intermediate transit-amplifying cells would lack self-renewal capabilities, but could continue to accumulate genetic changes and possibly a mutation conferring self-renewal capabilities to the cell or alternatively, these cells could also acquire CSC characteristics through microenvironmental stimuli. Moreover, cancer stem cell subclones during tumour progression can also accumulate additional epigenetic and genetic changes that could provide them with more advanced characteristics. Thus, the tumour represents a dynamic stage where clones of cancer stem cells continuously evolve, some clones in specific moments can become more superior to others in terms of metastatic potential or chemoresistance (Navin et al., 2011). Thus the representation of CSC clones in particular time is a snapshot of tumour growth. Whether the extent of heterogeneity found within CSCs suggest that future therapies should aim to target these multiple clones is still an open question but regardless of the answer, what is clear from the overwhelming amount of literature is a need to develop therapeutics that specifically target CSCs as they are clearly implicated in chemoresistance and disease relapse.

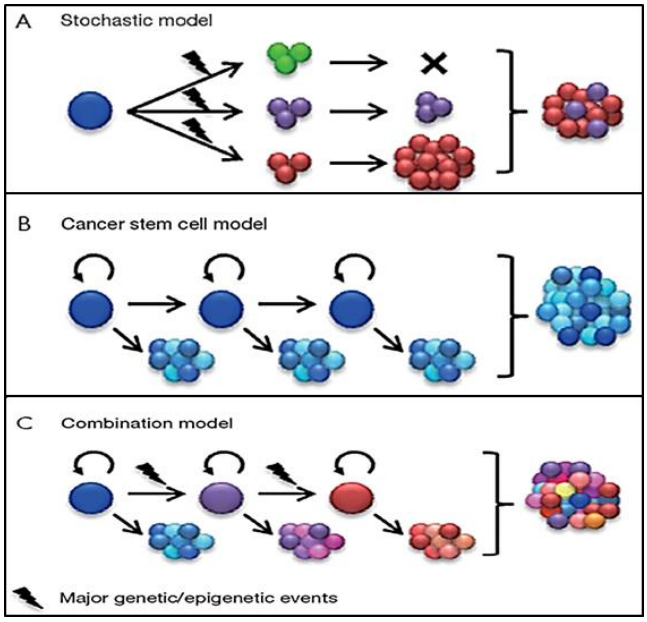

Figure I2. **(A)** The stochastic model indicates a clonal evolution of cancer**. (B)** The cancer stem cell model assumes that cancer is organized in a hierarchical structure; tumourigenic potential is limited to the CSCs and cellular heterogeneity of the cancer is a product of multipotent CSCs**. (C)** Combination model in which cancers are driven by one or multiple dominating CSC clones. *Adapted from (Wang et al., 2013a)*

2.2. Identification of cancer stem cells

Most of our understanding of CSCs has come from studies of hematopoietic malignancies. Furth and Kahn (Furth, 1935) were the first to suggest the existence of "CSCs". Using cell lines, they showed that a single leukemic cell was able, when transplanted into a mouse, to transmit the systemic disease. Robert Bruce and Hugo Van der Gaag (Bruce and Van Der Gaag, 1963) used spleen colony-forming assay (CFU-S) - a tool first developed by James Till and Ernest McCulloch (Becker et al., 1963; Buick et al., 1977) - to show that only a small subset of primary cancer tissue was able to proliferate *in vivo*. Collectively, these studies pointed towards the functional heterogeneity that exists within tumours - not every cell is able to proliferate to form a colony *in vitro* or to give rise to a tumour when transplanted *in vivo*, which introduced the concept of CSCs. Two important technologic advances had a major impact on the identification of cancer stem cells. The first was the development of fluorescence-activated cell sorting (FACS), and the second was the xenotransplantation assays for normal and leukemic human stem cells (Wang JC, 2001). In the early 1990s, Dick and colleagues, using limiting dilution analysis together with disease-initiation models, showed that when isolated from acute myeloid leukemia (AML) patients, only a small fraction of the tumour cells with a characteristic marker signature were able to establish leukemia

in recipient mice (Bonnet and Dick, 1997). Leukemic engraftment could only be initiated from $CD34^{+}CD38^{-}$ fractions. Moreover, the xenograft assay allowed measurement of the frequency of the initiating cell and it was found to be in the order of one per million tumour cells. Thus, CSCs were identified in *AML* (Lapidot et al., 1994). After their discovery in hematopoietic cancers, CSCs were hypothesized to also exist within solid tumours. This theory gained support when Al-Hajj and colleagues in 2003 isolated putative small population of CSCs from breast cancer, with the marker expression profile CD44+CD24−, typically expressed on normal mammary stem cells (Al-Hajj et al., 2003). When these CSCs were re-injected into immunodeficient mice, breast cancer arose in eight out of nine cases. Li *et al*., were the first to identify a population of cancer stem cells in pancreatic cancer (Li et al., 2007). By using CD44, CD24 and ESA (EpCAM) as separation markers, they showed that a triple positive population CD44+/CD24+/ESA+ of cells exhibited several important cancer stem cell characteristics, including 1) representing a minor population of cells (between 0.2% and 0.8%) and 2) potential to form tumours in 50% of animals with a few as 100 triple positive cells forming tumours that were histologically indistinguishable from the human tumours from which they originated. It is important to note that in this initial study CSCs were compared to their triple negative counterparts, CD44-/CD24-/ESA-. Since ESA (EpCAM) marks epithelial cells it is possible that EpCAM negative cells represented non-epithelial tumour cells, such as immune cells, stromal cells or cells of mouse origin. Using different cell surface markers, Hermann *et al*, demonstrated that CD133-positive cells form more tumours than CD133-negative populations (Hermann et al., 2007). Large numbers of CD133-negative cells could not induce tumour formation; however, small numbers of CD133-positive cells were found to be very tumourigenic. Fewer than 500 CD133-positive cells recapitulated tumours in mice. Another important finding of this study was that cells positive for CD133 and for CXCR4 exhibited a higher metastatic potential than other populations from the same tumours, supporting the observation that CXCR4 may be involved in tumour invasion and metastasis. While membrane bound cells surface markers are widely used to identify CSCs across different tumour entities, other assays based on functional properties are also valid means of identifying and isolating CSCs. For example, Van den Broeck *et al*., used a different method to study pancreatic CSCs (Van den Broeck et al., 2013), which involved isolating side population (SP) and non-side population (NSP) cells from PDAC surgical resection specimens using the Hoechst 33342 dye. Hoechst 33342 is a DNA dye historically used for flow cytometric analysis of the DNA content of live cells (Hamori et al.,

1980). Although Hoechst is able to penetrate intact cell membranes, it is actively transported out of cells by ATP-dependent ABC transporters (Zhou et al., 2001). Goodell and colleagues (Goodell et al., 1996) established that a small fraction of cells isolated from bone marrow barely expressed the dye and were characterized by the lack of fluorescence when analyzed by flow cytometry. These low-staining fractions of cells are now commonly known as the side population (SP). Van den Broeck *et al*., showed that the PDAC tumour SP fraction had higher expression of genes such as multidrug resistance transporter ABCB1 and also CXCR4 compared to the NSP. While SP has also been used by many other groups to isolate CSCs from other solid tumour such as breast cancer, its applicability in pancreatic cancer may be limiting. For example, Miranda-Lorenzo *et al*, recently showed that CSCs isolated from primary patient samples using SP were no more tumourigenic than NSP cells (Miranda-Lorenzo et al., 2014). Thus, the usefulness of SP as a means of isolating pancreatic CSCs is still under debate. Moreover, Miranda-Lorenzo *et al*, discovered and characterized a new specific marker for CSCs of diverse epithelial cancers, including PDAC. The study showed that freshly isolated epithelial cancer stem cells (CSCs) bear autofluorescent cytoplasmic vesicles. The membrane of these vesicles co-localize with the ATP-dependent ABCG2 transporter, also overexpressed in autofluorescent CSCs. Moreover, vitamin B2, riboflavin, which is a substrate for ABCG2 accumulates inside the intracellular vesicles, thus conferring luminosity to these cells (Miranda-Lorenzo et al., 2014). The authors also showed that autofluorescent cells displayed characteristics of CSCs, such as enhanced self-renewal properties, overexpression of pluripotency genes, increased invasiveness, and exclusive tumourigenic capacity in immune-deficient mice. From an isolation standpoint, autofluorescence allows these cells to be tracked in real time and in an easy and simple manner, paving the way for rapid screening assay to identify new drugs or drug combinations. Table I2 summarizes cancer stem cell markers for pancreatic cancer.

Pancreatic ductal adenocarcinoma	Marker	Reference
Tumour initiating cells	EpCAM+CD44+CD24+	Li et al.,2007
	CD133+	Hermann et al.,2007
	ALDH-1	Felderman et al.,2007; Jimeno et al.,2009; Rasheed et al.,2010
	Side population/ABCG2	Kabashima et al.,2009
	PD2	Vaz et al., 2014
	$\alpha_v\beta_3$	Seguin et al.,2014
	Autofluorescence	Miranda-Lorenzo et al.,2014
Migrating cancer stem cells	CD133+CXCR4+	Hermann et al.,2007

Table I2. Cancer Stem cell markers for pancreatic cancer. *Adapted from (Hermann et al., 2009)*

3. DNA METHYLATION

Classically, the development of cancer in humans has been viewed as a progressive, multistep process of transformation of normal cells into malignant cells driven by genetic alterations (Hanahan and Weinberg, 2000). A wealth of data, however, now indicate that human cancer cells, in addition to a large number of genetic alterations exhibit prominent epigenetic abnormalities, such as aberrant DNA methylation and histone modifications (Jones and Baylin, 2007). This knowledge has largely changed the view of cancer being a solely genetic disease. The structure of chromatin defines the way genetic information, in the form of DNA, is organized within the cell. This organization of the genome greatly influences the activation or silencing status of genes. Most of these changes are inheritable and are established during differentiation (Sharma et al., 2010). They are also stably maintained through multiple cycles of cell division, enabling cells to have distinct identities while containing the same genetic information.

Chromatin is made of repeating units of nucleosomes, which consist of ∼146 base pairs of DNA wrapped around an octamer of four core histone proteins (H3, H4, H2A and H2B) (Luger et al., 1997). There are four main epigenetic mechanisms that can influence the structure of chromatin: DNA methylation, covalent histone modifications, non-covalent mechanisms such as incorporation of histone variants and nucleosome remodelling and non-coding RNAs including microRNAs (miRNAs) (Bernstein et al., 2007). These modifications work together to regulate the function of the genome by altering the local structural dynamics of chromatin, primarily regulating its accessibility and compactness. The interplay of these modifications creates an ‘epigenetic landscape’ that regulates the way the mammalian genome manifests itself in different cell types, developmental stages and disease states, including cancer (Sharma et al., 2010).

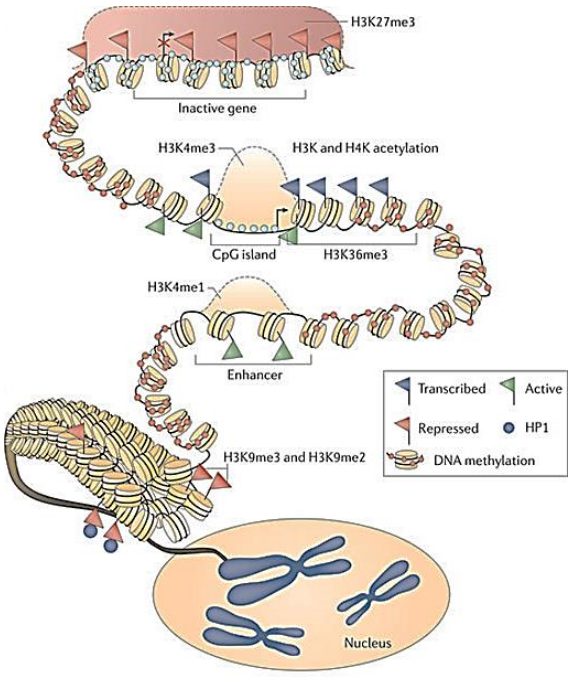

Figure I3. This diagram shows the balanced state of chromatin, nucleosome positioning and DNA methylation, which maintains the normal packaging state of DNA. *Adapted from (Baylin and Jones, 2011)*

The most extensively studied epigenetic modification in mammals is DNA methylation. It allows stable gene silencing and in association with histone modifications and other chromatin associated proteins, plays very important role in regulating gene expression and chromatin architecture (Sharma et al., 2010). While non-CpG methylation has been reported in pluripotent cells (Arand et al., 2012), in mammals DNA methylation occurs predominantly at CpG dinucleotides (Jones and Liang, 2009). CpG dinucleotides are not evenly distributed across the human genome but are instead concentrated in short CpG-rich DNA stretches called 'CpG islands' and regions of large repetitive sequences (e.g. centromeric repeats, retrotransposon elements, rDNA etc.) (Bird, 2002). A CpG island is defined as a sequence with a GC content that is greater than 55% and ratio of CpG to GpC of at least 0.65. CpG islands are at least 500 base pairs long (Takai and Jones, 2002) and they occupy ~60% of human gene promoters. While approximately 70% of CpG sites in the genome are methylated, the majority of CpG islands are protected from methylation and remain unmethylated during development and in differentiated tissue (Suzuki and Bird, 2008).

Various hypothesis were proposed to explain how DNA methylation contributes to gene silencing. For example, DNA methylation could create a physical barrier, blocking transcription

factors from accessing target-binding sites e.g. *c-MYC* and *MLTF*. Alternatively, it can provide binding sites for methyl-binding domain proteins, which can mediate gene repression through interactions with histone deacetylases (HDACs) (Jones et al., 1998; Nan et al., 1998). Thus, DNA methylation uses a variety of mechanisms to heritably silence genes and non-coding genomic regions.

The methylation reaction of cytosines is mediated by a class of enzymes called DNA methyltransferases (DNMTs), which catalyze the transfer of the methyl group from S-adenosyl-methionine onto cytosine (Figure I4). Five members of the DNMT family have been identified in mammals: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L (Kulis and Esteller, 2010). Among these, DNMT2 does not function as a methyltransferase for DNA but instead functions as a RNA methyltransferase (Goll et al., 2006). Likewise, DNMT3L does not possess the methyltransferase catalytic domain, leaving only DNMT1, DNMT3A and DNMT3B as true functional DNA methyltransferase (Aapola et al., 2000).

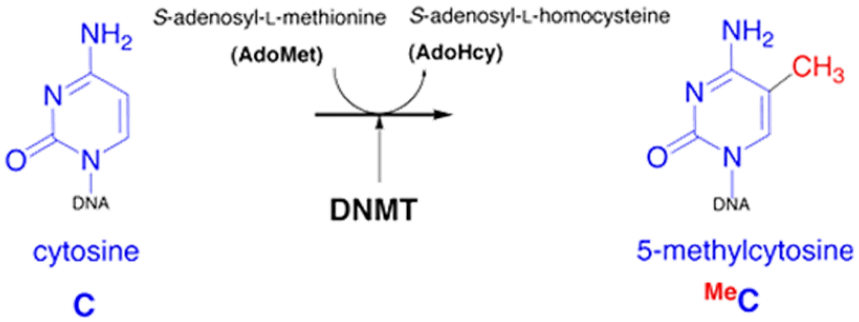

Figure I4. Schematic representation of the biochemical pathways for cytosine methylation, catalysed by the DNMT enzymes. *Adapted from* (Moison C, 2013)

After DNA replication, DNA methyltransferase 1 (DNMT1) is the principal enzyme responsible for maintenance of cytosine methylation at CpG dinucleotides (Kim et al., 2009). DNMT1 copies the present methylation patterns from the parental DNA strand to the newly synthesized strand (Chen and Riggs, 2011). Conversely, DNMT3a and DNMT3b, although also capable of methylating hemimethylated DNA, function primarily as de- novo methyltransferases to establish DNA methylation during embryogenesis (Okano et al., 1999).

3.1. DNA methylation in cancer

Cancer initiation and progression are followed by global changes in DNA methylation (Feinberg and Vogelstein, 1983) and it was discovered that the cancer epigenome is marked by genome-wide hypomethylation and site-specific CpG island promoter hypermethylation events (Jones and Baylin, 2002). DNA hypomethylation occurs at various genomic sequences including repetitive elements, retrotransposons or CpG poor promoters and introns. DNA hypomethylation can lead to the activation of growth-promoting genes, such as *R-Ras* in gastric cancer, and cyclin D2 and maspin in pancreatic cancer (Nishigaki et al., 2005; Oshimo et al., 2003). Regarding hypermethylation events in cancer, the first tumour suppressor gene (TSG) discovered to be silent by DNA methylation in cancer was retinoblastoma gene Rb (Greger et al., 1989). Since then, methylation specific silencing of other TSGs have been described in various cancers and these include *p16, MLH1* and *BRCA1* (Baylin, 2005). These genes are involved in various cellular processes including DNA repair, cell cycle, cell adhesion, apoptosis and angiogenesis.

In normal cells, the level of DNMT1 is tightly regulated during cell cycle (Szyf et al., 1991). Several studies during the last few years show that DNMT1 is up-regulated and hyperactive in some cancer types, including lung (Lin et al., 2007), colorectal (De Marzo et al., 1999), liver (Park et al., 2006) and gastric (Etoh et al., 2004) cancers. DNMT1 function could be regulated by transcriptional, post-transcriptional and post-translational mechanisms.

3.2. DNA methylation in cancer stem cells

Many studies have focused their attention on deciphering the genetic and epigenetic mechanisms that are responsible for the acquisition and preservation of CSC features. Since epigenetic mechanisms are involved in the regulation of transcription programs that control self-renewal and differentiation of adult stem cells, several studies indicate that establishment and maintenance of CSCs features can be orchestrated via a similar mechanisms (Balic et al., 2013; Hernandez-Vargas et al., 2011; Kaur et al., 2012). Moreover, the ability to predict which cells are tumourigenic based on marker expression indicates that the tumourigenic cells are intrinsically different from non-tumourigenic cancer cells. Yet, no clear morphological distinction was found between these two population of cells (Al-Hajj et al., 2003) implying that differentiation need not be so obvious for the cells to be hierarchically organized. The observation that CSCs are thought to be rare in the cancers found so far to follow a cancer stem cell model implies, that epigenetic

rather than genetic differences should allow rare CSCs to be functionally different than their non-CSCs counterparts. Moreover, epigenetic differences may have to be largely irreversible because if non-CSC could efficiently revert to a CSC state, it would not be possible to distinguish them and such cancers would not be hierarchically organized.

The importance of DNA methylation in the regulation of CSCs and tumour growth was first shown for leukemia stem cells. Trowbridge *et al.* showed that the development of leukemia was blocked by abrogation of DNMT1; haploinsufficiency of DNMT1 resulted in tumour suppressor gene derepression, impaired CSC self-renewal and delayed leukemogenesis (Trowbridge et al., 2012). Furthermore, additional studies were able to show differential methylation in specific CSC markers between tumour cell populations. Studies focused on breast cancer showed that CSC genes, such as *CD44*, *CD133* and Musashi-1(*MSI1*) are regulated by the methylation status of promoter CpG regions and that hypomethylation activates these genes in a clinically more aggressive subtype of breast cancer (i.e. triple negative breast cancer) (Baba et al., 2009; Kagara et al., 2012; Yi et al., 2008). Moreover, studies performed in pancreatic cancer comparing methylation profiles in invasive cancer cells and their non-invasive counterparts revealed significant correlation between the invasive property of pancreatic cancer cells and multiple pathways such as the NF-κB signalling pathway. Many genes identified, like *BIMP*, *TNFR* and *CD49* were methylated in the non-invasive fraction of the cells but demethylated in the invasive population (Sun et al., 2013a). In addition, the authors also found other candidate genes that play a role in multiple cancer models, such as *BMP4*, *GATA6* and *SOX9*.

All these studies suggested that difference in the DNA methylation level of specific genes could be responsible for their differential expression and subsequent role in gain and maintenance of CSC characteristics. Using techniques of higher genome wide coverage would definitively further expand the pool of potential candidate genes regulated by DNA methylation in CSCs, which could then be exploited to target candidates and potentially eliminate CSCs using epigenetically targeted therapy.

3. 3. Epigenetic therapy in cancer

Unlike genetic mutations, epigenetic changes are reversible, and as such, drugs that restore the epigenetic balance represent exciting potential therapeutic targets for cancer. The aim of epigenetic therapy is to reverse the alterations that occur in cancer and in that way restore the

'normal epigenome landscape'. In the past years various epigenetic drugs have been discovered and have shown success in reversing DNA methylation and histone modification aberrations in cancer (Yoo and Jones, 2006). Among the first epigenetic drugs proposed for use as cancer therapeutics were inhibitors of DNA methylation. The cytotoxic agents, 5-azacytidine (5-aza-CR) and 5-aza-2′-deoxycytidine (5-aza-CdR), induced inhibition of DNA methylation and differentiation of cultured cancer cells and these results highlighted their potential use as anti-cancer therapeutics (Constantinides et al., 1977). 5-Aza-CR (azacitidine) and 5-aza-CdR (decitabine) have now been approved by the US FDA for use in the treatment of myelodysplastic syndromes. The possible clinical uses of other improved DNA methylation inhibitors such as zebularine, which can be orally administered, is currently under investigation (Cheng et al., 2004). The advantage of Zebularine is that this drug is more stable and less toxic, and like aza-CR and 5-Aza-CdR, Zebularine incorporates into DNA and forms a covalent irreversible complex with DNMTs (Marquez et al., 2005). A list of DNMT inhibitors and the status with respect to clinical use is summarized in Table I3.

DNMT INHIBITORS IN CLINICAL DEVELOPMENT				
Drug classification	**Compound Name**	**Indications**	**Developmental Phase**	**Reference**
Nucleoside Analog	5-Azacitydine	MDS,solid tumors,leukemia	FDA approved for MSD(2005)	Kaminskas, at al.,2005
	Decitabine	MDS,CML	FDA approved (2006)	Gore, at al.,2006
	Zebularine	Urinary bladder cancer	Preclinical	Chang, at al.,2004
	5-F-CdR	Oncology	Phase I	Guo, at al.,2010
Non-nucleoside analogs	Procaine	Breast cancer, oncology	Preclinical	Cascamte, at al.,2014
	Procainamide	Prostate cancer, oncology	Preclinical	Lee, at al.,2005
	Hydralazine	Oncology	Phase III	Coronel,at al.,2011; Zambrano, at al.,2005
	RG108	Oncology	Preclinical	Stresemann at, at al.,2006
	SGI-1027	Oncology	Preclinical	Datta, at al., 2009
	EGGG	Oncology	Phase II	Nandakumar, at al., 2011

Table I3. Example list of DNMT inhibitors in clinical development.
Adapted from (Zheng, 2015)

In contrast to their promising results in hematological malignancies, DNMT inhibitors failed to show such promising results in solid tumours. A phase I study in melanoma and renal cell carcinoma where decitabine was combined together with interleukin-2, showed that decitabine did not alter the tolerability of IL-2, but caused grade 4 neutropenia in most patients (Gollob and Sciambi, 2007). There are different reasons for the limiting efficacy of demethylating agents in

solid tumours, such as 1) these drugs are replication dependent; therefore, they are likely to be less effective in cells that are proliferating at a lower rate (e.g. CSCs), 2) these drugs can have a global hypomethylating effect and apart from reactivating tumour suppressor genes they might also activate silent oncogenes in different cancer (Wang et al., 2012) and 3) the main reason for the slow progress towards introducing DNMT inhibitors as treatment options for solid tumours is their high toxicity. Both decitabine and azacytidine in hematological malignancies show effects at doses lower than those required for similar demethylation effects in solid tumours (Karahoca and Momparler, 2013). That is why the studies where low-doses of these inhibitors were used in non-hematological tumours are of great interest. A study done by Tsai *et al*., showed that short term exposure of cells to low (relatively non-toxic) doses of these agents could induce durable antitumour effects on hematological malignances but also in epithelial tumour cells (Tsai et al., 2012). Moreover, in a phase II trial, pretreatment with low-doses of azacytidne was found to alter the methylation of genes and cancer pathways, restoring the sensitivity to carboplatin in patients with ovarian cancer (Matei et al., 2012). Newer DNMT inhibitors, such as zebularine, or SGI-1027 are very promising candidates for clinical application because of their low toxicity and antiproliferative activity in cell lines and they are already being tested in the preclinical setting (Cheng et al., 2003; Datta et al., 2009).

3.4. Methods in DNA methylation profiling

As previously discussed, DNA methylation has a very important role in the regulation of gene expression, thereby the ability to assess the methylation status at the genome wide level should greatly facilitate our understanding of this layer of gene regulation in all aspects of cell biology; in normal as well as in the malignant setting. In this context there is a great interest in developing DNA methylation profiling technologies.

The discovery that treatment of denaturated DNA with sodium bisulfite deaminates unmethylated cytosines much faster than methylated cytosines brought a revolution to DNA methylation analysis in the 1990s. This chemical treatment of DNA turns an epigenetic difference into an actual genetic difference, because unmethylated cytosines become converted to thymidines (by uracil) (Zilberman and Henikoff, 2007). Even though sequencing based methylation analyses are far more informative than other techniques, cost still remains a rate limiting step for many

researchers, especially in the context of large sample size studies. In this regard, array-based profiling approaches represent very common alternatives for genome-wide DNA methylation studies, allowing analysis of numerous samples at affordable costs. Illumina has developed methylation bead arrays as an outgrowth of their genotyping methods (Bibikova et al., 2006; Fan et al., 2006). This array was developed to provide single-base resolution, although two or more closely spaced cytosines may have to be analyzed together. The strength of this technique is that it provides quantitative evaluation of specific cytosines and can be used for processing many samples in parallel. Illumina Infinium HumanMethylation450 BeadChip (450K) is based on the Infinium Technology and contains more than 480,000 probes, targeting 99% of genes and 96% of CpG island regions (Figure I5) (Bibikova et al., 2011). The array provides coverage of a total of 21,231 out of 21,474 UCSC RefGenes with a global average of 17.2 probes per gene region. Multiple transcripts of RefSeq genes are included, plus additional genes and transcripts not covered by the UCSC database (total of 29,246 transcripts).

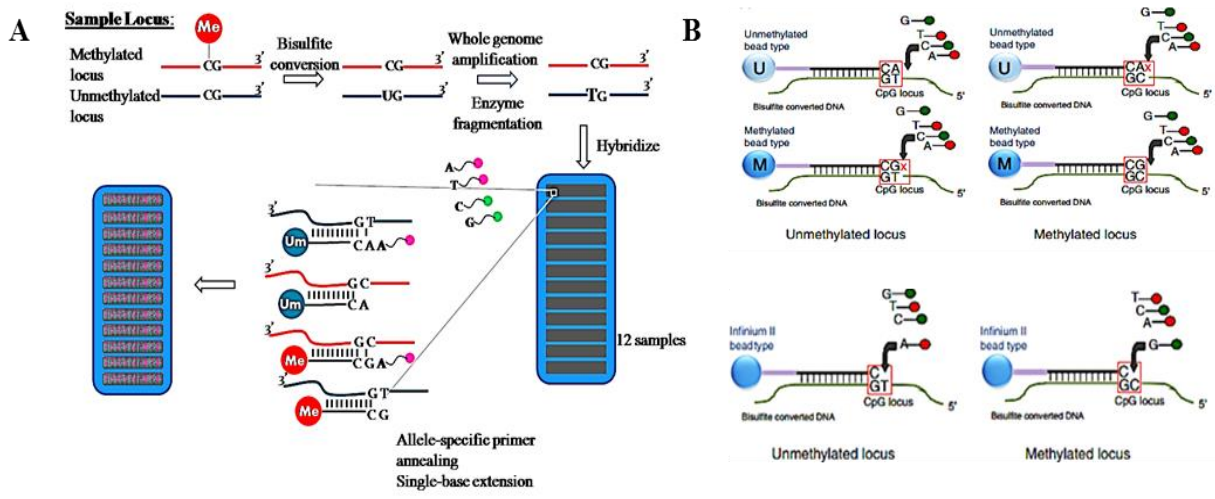

Figure I5. (A) Schematic representation of different steps in Illumina microarray technology**. (B)** Different types of probes often used in Infinium array. *Adapted from (http://www.illumina.com/pages.ilmn?ID=243)*

In this technique a small amount of genomic DNA (0.5–1 µg) is first treated with bisulfite, and then whole-genome amplified (WGA), enzymatically fragmented and purified before hybridization to specific BeadChips. During hybridization, the DNA molecules anneal to locus-specific probes immobilized onto individual beads. Two bead types correspond to two alleles of each CpG locus—one to the methylated (C) and the other to the unmethylated (T) state. Allele

specific primer annealing is followed by single-base extension. After extension, the array is fluorescently stained, scanned, and the intensities of the unmethylated and methylated bead types are measured. DNA methylation levels (beta values) are calculated for each CpG site in each sample as ratio of the signal intensity from the methylated bead type to the total locus intensity.

4. DNA DEMETHYLATION

4.1. Role of TET proteins in cancer

Historically, DNA methylation in general was considered to be a very stable chromatin modification. However, studies looking at global distribution of this modification through embryonic development discovered global loss of DNA methylation in early zygotes, especially in the male pronucleus (Messerschmidt et al., 2014). High-resolution genome-wide mapping of methylation in pluripotent and differentiated cells also confirmed the dynamic nature of this modification, indicating the existence of an active enzymatic process within mammalian cells that either erases or alters this chromatin modification (Baylin and Jones, 2011). Recent findings demonstrated that the ten-eleven translocation (TET) family proteins, including TET1, TET2 and TET3 (Figure I6), function as iron and α-ketoglutarate dependent 5-methylcytosine dioxygenases that convert 5-methylcytosine (5mC) bases to 5-hydroxymethylcytosine (5hmC) bases (Ito et al., 2010; Tahiliani et al., 2009), allowing the active process of demethylation. The three TET proteins have distinct expression patterns, suggesting possible non overlapping functions. TET1 and TET2 are highly expressed in embryonic stem cells (ESC), TET2 is also abundant in hematopoietic cells, and TET3 is present in oocytes (Gu et al., 2011) . In addition to the roles of TET-driven DNA modification in ESC and neuronal systems, emerging evidence suggests that TET-dependent DNA demethylation plays a role in tumourigenesis.

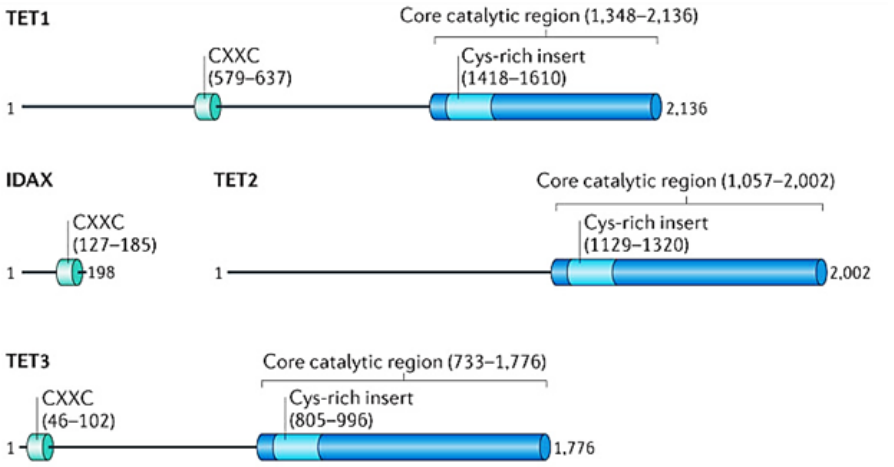

Figure I6. Three TET proteins are found in the mammalian genome. This figure shows different regions of proteins and points out that TET2 is different to other members as it lacks the CXXC domain, which is actually a part of another protein called IDAX. *Adapted from* (Pastor et al., 2013)

Large scale whole-exome sequencing studies by many groups have confirmed that TET2 is one of the most frequently mutated genes in chronic myelomonocytic leukemia (CMML; 50%), acute myeloid leukemia (AML; 20%), and myelodysplastic syndromes (MDS; 20%). A quick glance at The Cancer Genome Atlas (TCGA) database yields the general impression that all three TET genes are mutated in solid tumours, albeit at lower frequencies than observed for TET2 in hematological malignancies. For instance, somatic mutations in all three TET proteins have been reported in colorectal cancer (CRC) , and TET2 mutations and/or deletions have been observed in clear-cell renal cell carcinoma (ccRCC; 16%) (Sato et al., 2013), as well as in metastatic castration-resistant prostate cancer but not primary prostate cancers (Nickerson et al., 2013). Several studies have demonstrated a close correlation between decreased 5hmC levels and/or TET expression and robust tumour growth and metastasis, supporting the idea that TET proteins might serve as tumour suppressors in certain types of solid tumours (Hu et al., 2014; Song et al., 2013b).

OBJECTIVES

Pancreatic cancer is one of the most aggressive types of cancer and it has become apparent that pancreatic cancer is not only a genetic disease, but also an epigenetic disease characterized by widespread and profound alterations in DNA methylation. Recently it has been demonstrated that pancreatic cancer contains a subpopulation of autofluorescent CSCs that are essential for the maintenance of tumour progression, metastatic spread and for the chemoresistant to standard chemotherapeutic treatments (Miranda-Lorenzo et al., 2014). However, the mechanisms that govern self-renewal and tumourigenicity of cancer stem cells remain poorly understood. Convincing evidence suggests that the genome undergoes major epigenetic alterations during mammalian development and cell differentiation. Similar changes in the epigenetic signature during tumourigenesis may also, at least in part, explain many of the diverse properties that are commonly associated with tumour cell growth, invasion, metastasis and therapeutic resistance. Moreover, epigenetic differences may also explain the hierarchical organization of tumours and more specifically explain the basis for the apparent functional differences between CSCs and their progenies. Thus, our hypothesis is that, CSCs derived from the same clone in a tumour should harbour the same genetic alterations found in the bulk tumour cells, so that only differences in their epigenetic profile/machinery could explain their apparent biological differences.

The objective is to define the epigenetic landscape of CSCs versus non-CSCs by addressing the following aims:

1. To determine whether promoter methylation status regulates stemness genes in PaCSC
2. To perform genome-wide DNA methylome studies in order to identify and characterize candidate genes driving the CSC phenotype
3. Target DNA methylation machinery in order to contract CSC self-renewal and tumourigenic properties
4. Study the role of demethylase, specifically TET2 in PDAC CSCs

OBJETIVOS

El cáncer de páncreas es uno de los cánceres más agresivos, con profundas alteraciones no sólo genéticas sino también epigenéticas. Recientemente, nuestro grupo ha demostrado que el cáncer de páncreas contiene una subpoblación de células madre tumorales (CSCs) autofluorescentes, que son esenciales para la progresión tumoral, metástasis y resistencia a quimioterapias habituales (Miranda-Lorenzo et al., 2014). Sin embargo, los mecanismos que dirigen la autorenovación y tumorigenicidad de las CSCs son poco conocidos. Diversas evidencias sugieren que el genoma sufre alteraciones epigenéticas importantes durante el desarrollo en mamíferos y la diferenciación celular. Cambios similares observados en el proceso de tumorigénesis pueden explicar, al menos parcialmente, varias de las propiedades asociadas a este proceso como el crecimiento tumoral, metástasis y quimiorresistencia. Además, las diferencias en el epigenoma podrían explicar la organización jerárquica de los tumores y las diferencias funcionales entre CSCs y no-CSCs, ya que comparten el mismo perfil genético. Por lo tanto, nuestra hipótesis es que, CSCs derivadas del mismo clon en un tumor deberian albergar las mismas alteraciones genéticas encontradas en las células tumorales, de manera que sólo diferencias en su perfil epigenético / maquinaria podrían explicar sus diferencias biológicas aparentes.

El objetivo es definir el paisaje epigenético de CSCs versus no-CSCs, abordando los siguientes objetivos:

1. Determinar si los genes de pluripotencialidad se regulan por metilación de sus promotores en CSCs de páncreas
2. Realizar un estudio del estado de metilación del genoma completo, para identificar y caracterizar genes que potencialmente estén implicados en el fenotipo CSC
3. Atacar la maquinaria celular encargada de la metilación, para inhibir la autorenovación y propiedades tumorigénicas de las CSC.
4. Estudiar el papel de la demetilasa TET2 en CSCs pancreáticas.

MATERIALS AND METHODS

1. MICE

1.1. Study approval

Mice were housed in the CNIO's animal facility in accordance with institutional policies and federal guidelines. Animal treatments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain). Human pancreatic tumours were obtained with written informed consent and after approval from the Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain).

1.2. Xenograft

PDAC xenografts, generated from patient-derived samples, were kindly obtained from Manuel Hidalgo's group (CNIO, Spain). Primary tumours were minced into small fragments and then implanted subcutaneously, in duplicate, in 4 to 5 nude mice (NU-*Foxn1*^{nu}*;* Charles River, Wilmington, MA, USA). Once tumours reached 1cm^3, they were resected, minced and re-implanted in another set of female nude mice, following the protocol described in Rubio-Viqueira *et al*. (Rubio-Viqueira et al., 2006) and illustrated in Figure M&M1.

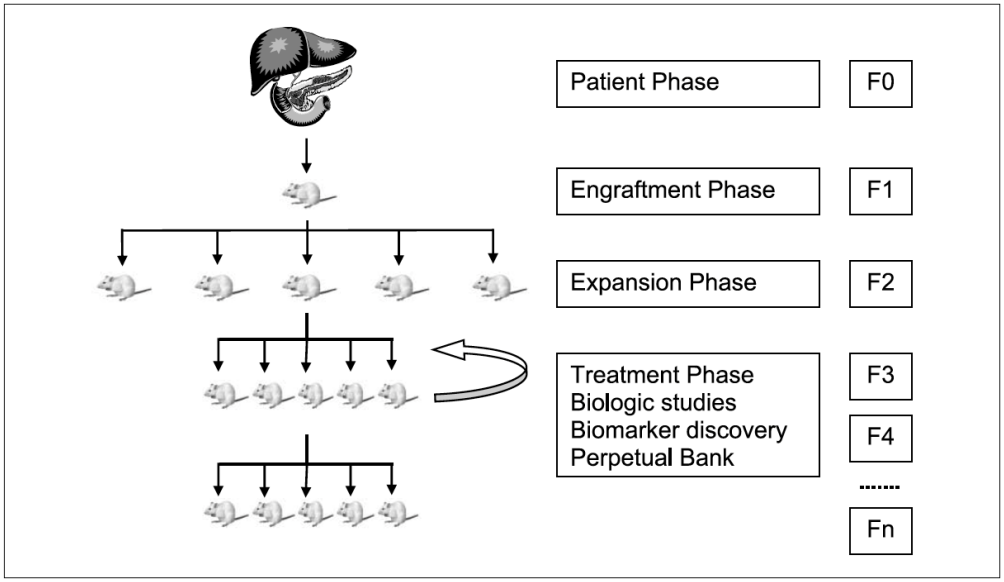

Figure M&M1. Xenograft study scheme. Tumour samples are implanted in F1 generation mice and then expanded in a cohort of nude mice. *Adapted from (Rubio-Viqueira et al., 2006)*

1.3. In vivo tumourigenicity

Primary pancreatic cancer sphere derived cells or adherent cells sorted for GFP Lenti-ShTet2 vector were used as detailed below. For tumourigenicity assays, serial dilutions (e.g. 10^3, 10^2) of single-cells resuspended in Matrigel™ (BD Bioscience, Heidelberg, Germany) were subcutaneously injected into right and left flank of female nude mice.

2. CELL CULTURE

2.1. Primary human cancer cells

Primary pancreatic cancer tumours were minced, mechanically (gentleMACS Dissociator; Miltenyi, Bergisch-Gladbach, Germany) and enzymatically digested with collagenase for 60 min at 37°C followed by a centrifugation for 5 min at 1,200 rpm (Mueller et al., 2009) (Stem Cell Technologies, Vancouver, BC). Cell pellets were resuspended and cultured in RPMI (Invitrogen, Alcobendas, Spain) supplemented with 10% FBS and 50 units/ml pen/strep.

2.2. Treatments

Primary sphere derived human PDAC cells were treated with zebularine (75μM) for 7days. The drug was added every 2nd day directly into cell suspension.

2.3. Antagomirs

Knockdown of miR-17-92 was achieved *in vitro* administering miR-17, 18a, 19a, 19b, 20 antagomir mix or scrambled control were chemically synthesized as 2-O-methyloligoribonucleotides by BioSpring (Frankfurt, Germany). The antagomirs are labelled with Cy3 and contain cholesterol that facilitate the entry into the cells and allow tracking them.

2.4. Cell viability assay

Cells were seeded in 96-well plates (Nalgen Nunc International, Penfield, NY) at a concentration of 10^4 cells per well in 100μL of complete medium. Cells were incubated for 24 hours after administration of compounds to allow an optimal attachment. Cytoxicity was measured using a bioluminescence-

based assay (The Toxilight BioAssay Kit, Lonza) that measures adenylate kinase (AK) released from damaged cells, as per the manufacturer's instructions. Briefly, 20 µl of supernatant was collected on indicated days and transferred to white 96-well plates (BD Biosciences). 100µl of AK detection reagent was then added to each well, and luminescence (RLU) was measured using luminometer following the manufacturer's instructions. Each experiment was carried out with three replicate wells for all conditions tested, and all the experiments were done in triplicate.

2.5. Sphere formation assay

Spheres were generated by culturing ~2×10^3 pancreatic cancer cells in Ultra-Low attachment plates (Corning, USA) in suspension in serum-free DMEM/F12 supplemented with B27 (1:50, Invitrogen, Alcobendas, Spain), 20 ng/ml bFGF and 50 units/ml pen/strep for a total of 7 days, allowing spheres to reach a size of >75µm. For serial passaging, 7-day-old spheres were filtered using 50µm cell strainers, dissociated into single cells, and then re-cultured for 7 additional days as previously described (Lonardo et al., 2011)

2.6. Colony assay

Cells were seeded in 6-well plates (Corning, USA) at a concentration of 10^3 cells per well in 2ml of complete medium (RPMI medium (Roswell Park Memorial Institute), 10% fetal bovine serum (FBS) and 50 units/mL penicillin/streptomycin). After two weeks cells were washed twice with phospahate-buffer saline (PBS) and the remaining adherent cells were stained with crystal violet (0.2% in 2% ethanol) and analyzed spectrophotometrically, as described previously (Sanchez et al., 1996).

2.7. Wound healing assay

Confluent cultures of primary cancer cells seeded in a 6-well plate were scratched using a 1ml pipette. Cells were washed twice with PBS to remove cell debris and then incubated at 37°C in RPMI medium (Roswell Park Memorial Institute), supplemented with 10% fetal bovine serum (FBS) and 50 units/mL penicillin/streptomycin. Migration was evaluated 24 h later by calculating the average size of the wound determined by measuring the size of the wound at three locations (n = 3 wounds per cell/treatment).

3. FLOW CYTOMETRY

3.1. Flow cytometry analysis

For flow cytometry analysis, primary pancreatic cells, dissociated cells from spheres cultures or cells obtained from tumour digestions were stained using different combinations of antibodies. The following antibodies were used: anti-hCD133/1-APC or PE (Miltenyi Biotec); hEpCAM-APC, hCXCR4-APC, or appropriate isotype-matched control antibodies (all from BD, Heidelberg, Germany). DAPI was used for exclusion of dead cells. Cells were acquired with a FACS CANTO II instrument (BD, Heidelberg, Germany). Data were analysed with FlowJo 9.2 software (Tree Star, Ashland, OR).

3.2. FACS sorting

Primary pancreatic cells, dissociated cells from sphere cultures or cells obtained from tumour digestions were adjusted to a concentration of 10^6 cells/ml in Sorting buffer (1X PBS; 3% FBS (v/v); 3mM EDTA (v/v)). DAPI was added to exclude dead cells at a concentration of 2mg/ml. Cells were sorted with a FACS Influx instrument (BD, Heidelberg, Germany).

3.3. Apoptosis assay

Cancer cells and CSCs were plated at $3x10^5$ cells/well in a 6-well multi-well and cultured in presence of Zebularine (75uM) for 3 days. Attached and floating cells were collected, respuspended and stained for cell-surface marker CD133 when indicated prior to staining with Annexin V (550474; BD Bioscience) after incubation with Annexin V binding buffer (556454; BD Pharmingen). Cells were then incubated with DAPI.

3.4. Cell cycle analysis

Cells were trypsinized, washed in PBS, centrifuged, and pellets were fixed in 200µl of 70% ethanol and stored at -20°C until use. Cells were centrifuged and pellets resuspended in 200µl of PBS, and 10µg/mL of RNAse A was incubated for 1 hour at 37°C. Subsequently, cells were stained with DAPI to perform cell cycle analysis using FACS CANTO II (BD) instrument. Data were analysed by FlowJO software.

4. PROTEIN ANALYSIS

4.1. Protein extraction and quantification

Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The cell lysate was centrifuged at 14,000 rpm and supernatant was collected. Protein lysates were quantified using a BCA Protein Assay Reagent kit (Pierce, Thermo Scientific).

4.2. Western Blot

Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). 50µg of protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 1X TBS containing 5% BSA (w/v), or 5% (w/v) milk and 0.1% Tween20 (v/v), incubated with a 1:1000 dilution of antibodies against, Nanog (D73G4; Cell signalling); α-Tubulin (#2144; Cell signalling) or bactin (Sigma), Dnmt1 (D63A6; Cell signalling), p21 (12D2; Cell signalling), Tet2 (21F11; Active motif), overnight at 4ºC, washed 3 times with 1X PBS containing 0.1% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rat or goat anti-mouse antibody (Sigma), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Amersham, Barcelona, Spain).

5. RNA ANALYSIS

5.1. RNA extraction from tissue or cells

Total RNA was isolated by the guanidine thiocyanate method using standard protocols (Chomczynski and Sacchi, 1987).

5.2. RT-qPCR

1 µg of purified RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Barcelona, Spain), followed by SYBR green RTqPCR using an Applied Biosystems

7500 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial 10 minute denaturation step at 95 °C followed by 40 cycles of denaturation (15 sec at 95 °C) and annealing/extension (1 min at 60 °C). The list of utilized primers are shown in Table M&M 1.

Gene	Primer sense	Primer antisense
NANOG	AGAACTCTCCAACATCCTGAACCT	TGCCACCTCTTAGATTTCATTCTCT
OCT3/4	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
SOX2	AGAACCCCAAGATGCACAAC	CGGGGCCGGTATTTATAATC
KLF4	ACCCACACAGGTGAGAAACC	ATGTGTAAGGCGAGGTGGTC
MAML3	CAGGGCACACTGAGCGAAGA	TGGATGCTGCTCCTGACCGT
DNMT1	CAGGAAGAACGGCCGCAGCA	AGGCTTTGCCGGCTTCCACG
DNMT3a	GAGGCACTTGACACCGGCCC	GATGGCTCCACCTGGCGCTG
DNMT3b	TGCGTCTTCGAGTCTTGTTCTCGTA	ACCCAACAACACGCAACCCGTG
P21	AGTTCCTTGTGGAGCCGGAGC	GACATGGCGCCTCCTCTGAGT
TGFBR2	CAACCACCAGGGCATCCA	TCGTGGTCCCAGCACTCA
ALK4	TGCAACAGGATCGACTTGAG	GGAGCGTCTTGTCTTTGGAG
SMAD2	TCCCAGCAGGAATTGAGCCACA	GTTCTGCTGGAGAGCCTGTGTCC
SMAD4	CAGCACCACCCGCCTATGCC	TGGAACACCAATACTCAGGAGCAGG
TET1	TTTCCCTGACAGCAGCAACA	ACCTGCAGCTGTCTTGATCG
TET2.1	GAATGTTTGCCAGCCTCGTT	CCGCTGAGTGATGAGAACAGA
TET2.2	AATGTTTGCCAGCCTCGTTC	CCCCGCTGAGTGATGAGAA
CD133	CAGAGTACAACGCCAAACCA	AAATCACGATGAGGGTCAGC
BACTIN	GCGAGCACAGAGCCTCGCCTT	CATCATCCATGGTGAGCTGGCGG

Table M&M1. List of primers used for RT-qPCR

5.3. RNA RT-qPCR

For miRNA analysis, 1µg of total RNA was reverse-transcribed using the NCode VILO miRNA cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). This step adds a polyadenylate tail to the miRNA population within the total RNA samples. The resulting cDNA was subjected to real-time PCR using SYBR Green ER qPCR Mix (Invitrogen). The Universal qPCR Primer was provided in the VILO kit and the forward primer for miR-17, 18a, 19a, 19b, 20a, 92, Snord95 and Snord44 were purchasing from Qiagen.

6. DNA ANALYSIS

6.1. DNA extraction from cells

Total DNA was isolated by the guanidine thiocyanate method using standard protocols (Chomczynski and Sacchi, 1987).

6.2. Bisulfite sequencing

Two fragments on human NANOG upstream region were analysed, they have been called NANOG-F1 and NANOG-F2, respectively. In brief, DNA samples from sphere-derived FACS sorted autofluorescent-positive and autofluorescent-negative cells were used for subsequent bisulfite conversion by EpiTect Bisulfite kit (Qiagen), as recommended by the manufacturer. NANOG-F1 (492bp) lies upstream from NANOG-F2 (593bp). Both fragments have been amplified with a nested PCR system. 30 cycles of PCR with an annealing temperature of 62ºC were performed for each PCR, with Platinum Taq DNA Polymerase (Invitrogen) under otherwise standard conditions. Primer sequences are provided below. PCR products were cleaned up on NucleoFast PCR plates (Macherey-Nagel) and cloned on pGemT-easy (Promega). 10-12 colonies were sequenced per sample and analysed with BiQ Analyzer software (Bock et al., 2005).

Gene	PCR step	Primer sense	Primer antisense
NANOG-F1	1st PCR primers	AATCATCCTATTTCCTACgAaACATAa	TtttAGGTtTGGTGATTTGTtA
	Nested PCR primers	CCTACgAaACATAaACTATCTaCCTa	tAGGTtTGGTGATTTGTtATGTt
NANOG-F2	1st PCR primers	CACCCTAATaAaAATTTCAATAACCTCAaa	tAGAAtGTTAAAATttTGGAGTtTt
	Nested PCR primers	TTCAATAACCTCAaaAATTTAAaaTaCATa	GAGTtTtTAGATTTATAATGAAGGtTt

Table M&M2. List of primers used for bisulfite sequencing

6.3. Illumina methylation array

HumanMethylation450K BeadChip (Illumina, Inc, San Diego, CA), using Infinium HD Methylation assay for genome-wide DNA methylation screening, was employed. In brief, genomic DNA (1µg) from each sample was treated by bisulfite conversion with the EZ DNA

Methylation Kit (catalog nº D5004, Zymo Research, Orange, CA) according to manufacturer recommendations. After bisulfite conversion, the standard protocol provided by Illumina was used for DNA methylation analysis. Briefly, bisulfite converted DNA was isothermally amplified at 37°C (20-24h). The amplified DNA product was fragmented by an endpoint enzymatic process. Fragmented DNA was precipitated, resuspended, and applied to an Infinium Human Methylation450K BeadChip and hybridized at 48°C (16-24h). During hybridization, the amplified and fragmented DNA samples anneal to specific oligomers that are covalently linked to the different bead types. The bead chips were then subjected to a single-base extension reaction. This reaction incorporates labelled nucleotides into the extended primers hybridized to DNA on the BeadChip. The HumanMethylation450K BeadChip applies both The Infinium I and The Infinium II assay chemistry technology. One or two probes are used to interrogate CpG locus, depending on the probe design for a particular CpG site. The Infinium I assay has two probes per site one "unmethylated" and one "methylated". The 3' end of each probe is designed to match either the protected cytosine (methylated design) or the thymine base (unmethylated design) resulting from bisulfite conversion and whole-genome amplification. The Infinium II assay has one probe per site. The 3' end of the probe complements the base directly upstream of the query site while single base extension results in the addition of a labeled G or A base, complementary to either the "methylated" C or "unmethylated" T.

6.3.1. Data processing

For methylation analysis, IDAT files were loaded into the R environment using the Bioconductor minfi package (Aryee et al., 2014). The arrays were then background and control normalized using the minfi package. Technical differences between Infinium I and Infinium II probes were removed using Subset-quintile Within-Array Normalisation, developed by Maksimovic *et al*. (Maksimovic et al., 2012) and available in the minfi package. The methylation status for each probe was recorded as a β-value that ranged between 0 and 1, where values close to 1 represent high levels of methylation and where values close to 0 represent low levels of methylation. Detection P-value was calculated for all probes on all arrays. P-value>0.01 indicates that the data point is not significantly different from background measurements. Probes were removed from analysis if in more than one samples had a detection P-value>0.01. Next, probes that are designed for sequences

on either the X or Y chromosome were removed. Finally, probes with single-nucleotide polymorphisms present within 10–50 bp from query site.

6.3.2. Differential methylation analysis

In order to assess differences in methylation between groups, the original β-values were converted to M-values via the logit transformation as recommended by Du *et al.* (Du et al., 2010). Differentially methylated probes were detected:

1) To obtain differently methylated probes in autofluorescent-positive and –negative cells from each tumour separately, we applied following approach. As only two biological replicate of autofluorescent-positive and -negative cells from each tumour were available, we first calculated average β-value of two biological replicate in autofluorescent-positive and autofluorescent-negative cells. Than we calculated delta β value (delta $\beta = \beta_{\text{value autofluorescent-positive}} - \beta_{\text{value autofluorescent-negative}}$), and filtered probes with delta $\beta > 0.05$. Next we identified unmethylated and methylated probes, such that unmethylated probes were once with β-value lower than 0.3 in both autofluorescent-positive and autofluorescent-negative cells. Similarly, we defined a probe as methylated if β-value was higher than 0.7 in both autofluorescent-positive and -negative cells. Moreover, a probe was classified as hypermethylated in autofluorescent-positive population if β-value was higher than 0.7 in autofluorescent-positive and lower than 0.7 in autofluorescent-negative (same criteria was used to define hypermethylated probes in autofluorescent-negative population). Conversely, the probe was defined as hypomethylated in autofluorescent-positive population if β-value was lower than 0.3 in autofluorescent-positive and higher than 0.3 in autofluorescent-negative (same criteria was used to define hypomethylated probes in autofluorescent-negative population).
2) Or to identify differently methylated probes in paired autofluorescent-positive and – negative cells from all tumours we used limma package (Smyth, 2005). Probes were considered to be differentially methylated if the resulting adjusted P-value was <0.05. The Benjamini–Hochberg method was used to adjust the P-values and ensure that the false discovery rate was <0.05.

6.4. Dot blot assay

For dot blot analysis we followed standard protocol. Briefly, DNA samples were diluted with TE buffer, denaturated by heating on 99°C for 5min, chilled rapidly on ice and then loaded on a Hybond N+ nylon membrane (GE Health, Piscataway, NJ, USA). DNA was cross-linked by the Hoefer™ UVC 500 Ultraviolet Crosslinker (70 000 micro-joules/cm2). After crosslinking membranes were blocked by 5% non-fat milk for 1 h at room temperature, incubated with a polyclonal anti-5hmC antibody (Active Motif, Carlsbad, CA, USA; #39769, 1:10,000) at 4°C overnight. 5hmC was visualized by chemiluminescence.

7. IMMUNOCHISTOCHEMISTRY

For histopathological analysis, FFPE blocks were serially sectioned (3 μm thick) and stained with haematoxylin and eosin (H&E).

8. LENTIVIRUS

To construct the lentiviral vectors carrying 2 different shRNAs targeting MAML3, a forward and a reverse oligonucleotide for each shRNA was designed, in order to reconstitute the shRNAs through a hybridization PCR and introduce restriction sites at the 5' and 3' of the shRNA for posterior cloning. The oligonucleotides are shown in Table M&M3. The shRNAs were cloned in the lentiviral vector pLVX-shRNA2 from Clontech, under the control of the U6 promoter and with mCherry as a transduction control. Replication-incompetent lentiviral particles were produced by calcium-phosphate transfection of HEK293T cells using the packaging plasmids pMD.2G (VSV-G) and pPAX2, as well as either one of the shRNA plasmids as shuttle vectors. The medium was replaced for fresh DMEM complete 6h after transfection, and 48h afterwards the medium was collected, cleared by low-speed centrifugation, filtered through 0.45 mm pore-size PVDF filters, and stored in aliquots at -80ºC. Subsequently the viruses were titered by flow cytometry analysis of mCherry expression in 293T cells infected with increasing dilutions of virus. For infection of primary PDAC cells, a multiplicity of infection of 2.5 i.u./cell was used and later sorted for mCherry.

shRNA	Sequence
MAML3.1 FW	GATCCACCGGCGGTGCTGATGTATATGTAGTTAGGTACCATAGCTACATATACATCAGCACCGTTTTTT
MAML3.1 RV	CGAAAAAACGGTGCTGATGTATATGTAGCTATGGTACCTAACTACATATACATCAGCACCGCCGGTG
MAML3.2 FW	5'GATCCACCGGAAGCTGTTCTCAGACATTAGTTAGGTACCATAGCTAATGTCTGAGAACAGCTTTTTTTT
MAML3.2 RV	CGAAAAAAAAGCTGTTCTCAGACATTAGCTATGGTACCTAACTAATGTCTGAGAACAGCTTCCGGTG

Table M&M3. ShMAML3 sequence

GIPZ Lentiviral Human TET2 shRNA *(RHS4430-200190653)* and control (*RHS4346)* were purchased from Dharmacon. Replication-incompetent lentiviral particles were produced by calcium-phosphate transfection of HEK293T cells using the packaging plasmids pMD.2G (VSV-G) and pPAX2, as well as the GIPZ shTET2 as the shuttle vector. The medium was replaced for fresh DMEM complete 6h after transfection, and 48h afterwards the medium was collected, cleared by low-speed centrifugation, filtered through 0.45 μm pore-size PVDF filters, and stored in aliquots at -80ºC. Subsequently the viruses were titered by flow cytometry analysis of GFP expression in 293T cells infected with increasing dilutions of virus. For infection of primary PDAC cells, a multiplicity of infection of 2.5 i.u./cell was used and later sorted for GFP.

8. STATISTICAL ANALYSES

Results for continuous variables are presented as means ± standard deviation unless stated otherwise and significance was determined using the Mann-Whitney test. All analyses were performed using SPSS 22.0 (SPSS, Chicago, IL). If not otherwise stated, significance is given as $p<0.05$

RESULTS

1. CHARACTERIZATION OF CANCER STEM CELLS IN PANCREATIC CANCER AND ESTABLISHMENT OF MONOCLONALY DERIVED TUMOURS

For our studies we used primary cell cultures derived from xenografts of primary human patient-derived pancreatic adenocarcinomas (A6L, 185, 354, 215 and 253) that have been previously described (Jones and Wagers, 2008; Rubio-Viqueira et al., 2006). Importantly, all cells for *in vitro* experiments were freshly isolated from early passaged xenografts and cultured as adherent cells (monolayer) or sphere-derived cells at low passages. There are different strategies used to identify and isolate cancer stem cells from pancreatic cancer tumours. For example CSCs can be enriched for by *in vitro* growth as spheres in anchorage-independent growth conditions that promote apoptosis of differentiated non-CSCs and thus subsequent enrichment for non-differentiated CSCs (Hermann et al., 2007). Other strategies rely on characteristics associated with normal stem cells, including the expression of particular cell-surface markers such as pentaspan transmembrane glycoprotein CD133, also known as Prominin-1 (Hermann et al., 2007), or CD44, CD24 and epithelial-specific antigen (ESA; also called epithelial cell adhesion molecule or Ep-CAM). More recently, Miranda-Lorenzo *et al*., identified an autofluorescent subcellular vesicle within CSCs that can be used to efficiently identify and isolate CSCs across numerous epithelial tumours independent of the expression of cell surface markers or functional properties, such as dye exclusion associated with side population (Miranda-Lorenzo et al., 2014).

The present thesis is based on the study of epigenetic marks specific to CSCs from PDAC. Thus, it was essential to first determine what methodology would best enrich for CSCs from primary xenograft-derived tumours. To do so, we first characterized populations of cancer stem cells from primary xenograft-derived pancreatic cell cultures using the aforementioned strategies: sphere culture conditions, expression of cell-surface CSC markers and autofluorescence to determine what methodology, or combination of methodologies would most efficiently allow for the isolation of PDAC CSCs.

Bulk tumours contain heterogeneous populations of cells. Apart from more differentiated tumour cells, which represent the majority of the cancer cells present in the tumour, there exists small sub-populations of cancer stem cells within the tumour. It is very unlikely that cancer stem cells are homogenous, instead, the tumour is believed to contain several clones of different cancer stem cells populations and their progenies (Gerlinger et al., 2012). For this reason, there is great

interest in generating model systems devoid of multiclonality, such as tumours derived from one single cell in order to better study the CSCs at the single cell level.

1.1. Different strategies used to characterize and isolate CSCs in primary PDAC

As previously shown (Cioffi et al., 2015; Lonardo et al., 2011) pancreatic cancer stem cells can be enriched for by *in vitro* anchorage-independent growth, which promotes the formation of structures called spheres. In these conditions more differentiated cells will undergo apoptosis and die, while cancer stem cells will undergo self-renewal, maintaining the stem cell pool and giving rise to differentiated progenies and/or more CSCs (Figure 1A). To characterize sphere-derived cells at the genetic level, we compared the expression of the pluripotency-associated genes *NANOG, OCT3/4, SOX2,* and *KLF4* in sphere cultures versus adherent (monolayer) cells by real-time PCR. This group of genes has been extensively described as the "core transcription network" in the regulation of pluripotency in human and mouse Embryonic Stem Cells (ESC) and Induced Pluripotent Stem Cells (iPS) (Maherali and Hochedlinger, 2008; Takahashi et al., 2007; Yamanaka and Takahashi, 2006). Since CSCs have been shown to be transcriptionally "similar" to ESCs, the evaluation of the expression of this core groups of genes in CSCs has become a routine means of measuring the "stemness" of putative CSCs populations. As expected and shown in Figure 1B, expression of pluripotency-associated genes was significantly higher in first generation spheres (seven days old) versus 70% confluent adherent cultures. In addition, we evaluated the protein expression of Nanog by western blotting and confirmed the up regulation of Nanog at the protein level in sphere cultures compared to adherent cultures (Figure 1C).

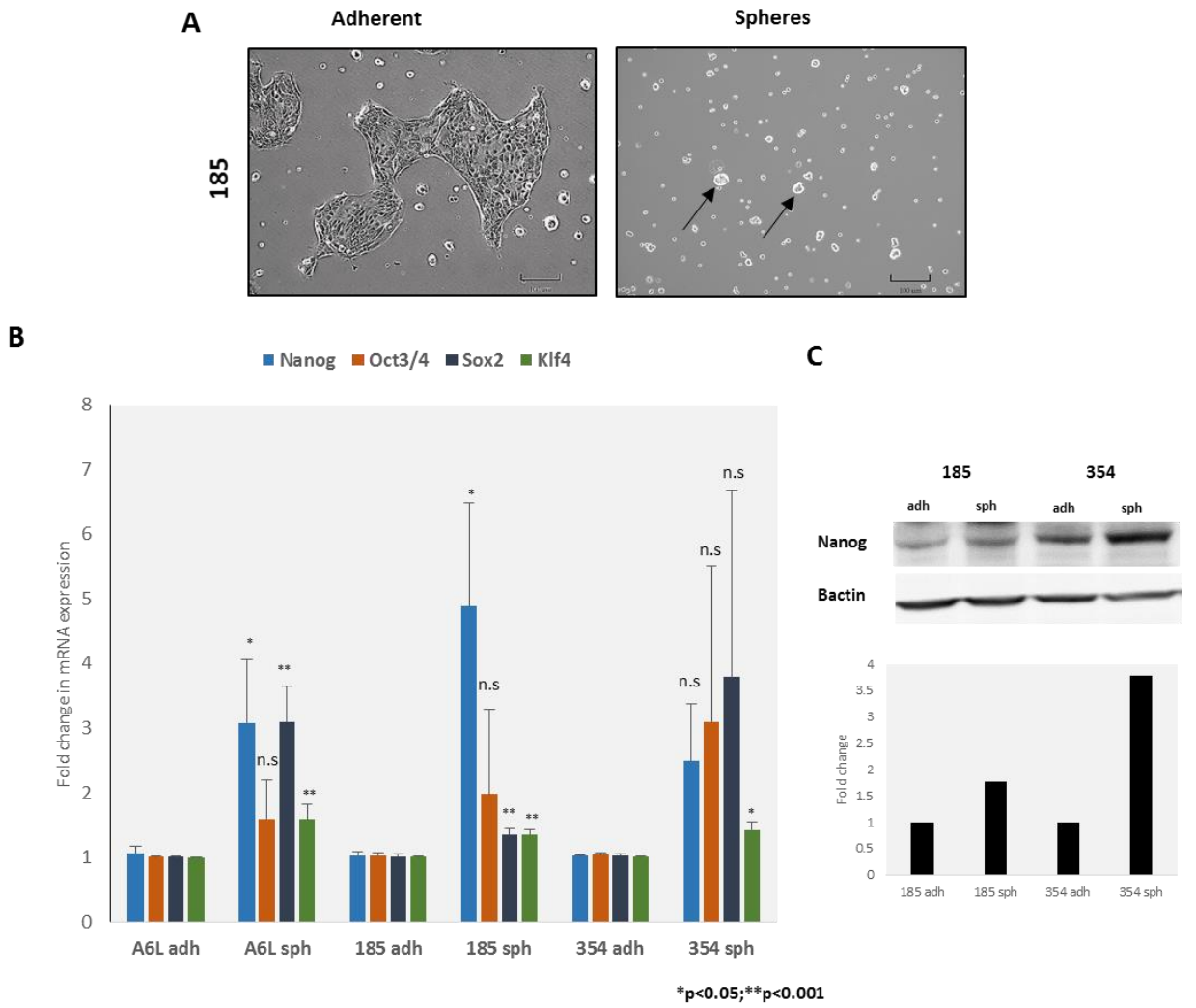

Figure 1. (**A**) Representative pictures of primary PDAC 185 cells in adherent (adh) and in spheres (sph) culture conditions. Pictures were taken when spheres were 7 days old (1st generation). (**B**) RTqPCR analysis of pluripotency-associated genes in primary PDAC cells A6L, 185 and 354 in 1st generation spheres versus adherent cells. Data are normalized to β-actin and represented as fold change in compare to adherent cells. **(C)** Western blot analysis of the pluripotency-associated gene NANOG in primary PDAC 185 and 354 spheres versus adherent cells. Protein levels were normalized to β-actin and subsequently quantified by densitometry. Change in protein level are represented as fold change in compare to adherent cells.

Next we characterized adherent- and sphere-derived cells in terms of cell-surface marker expression by flow cytometry, and as previously reported spheres were enriched in CD133-positive cells (Figure 2A). Moreover we sorted sphere-derived cells for CD133 by FACS sorting, and we assessed the expression of the pluripotency-associated gene *NANOG*. Expression of *NANOG* was higher in CD133-positive cells compared to CD133-negative cells at the mRNA (via RTqPCR analysis) and protein level (via WB analysis) (Figure 2B).

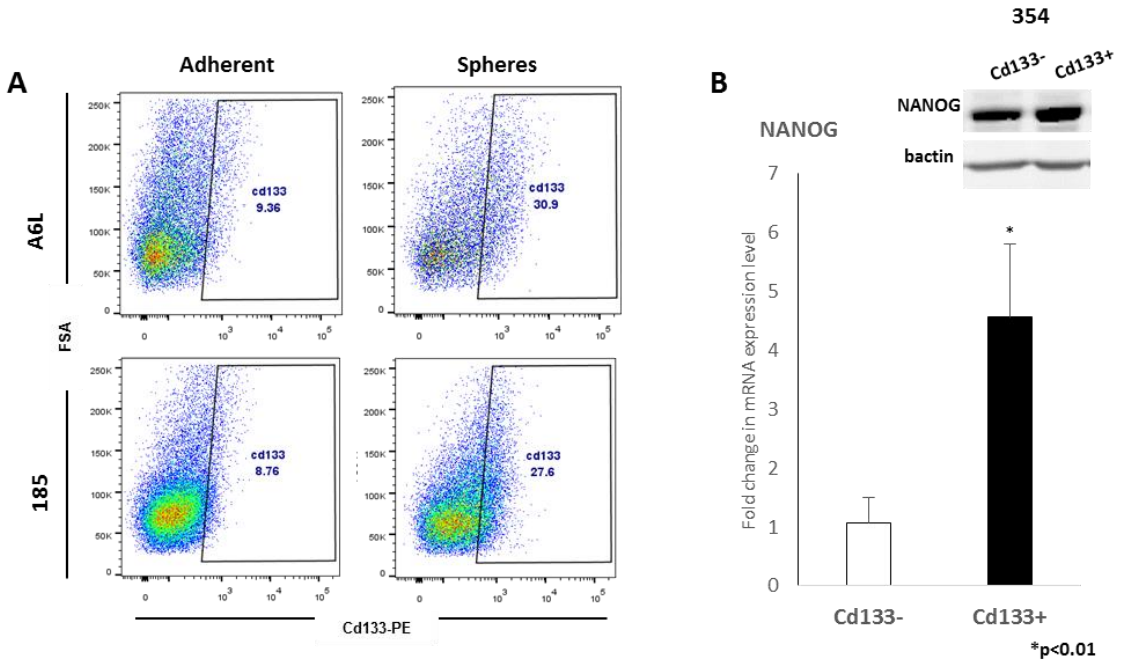

Figure 2. (**A**) Flow cytometry plots of CSC marker CD133 expression in 1st generation spheres and adherent cells from primary PDAC cells A6L and 185. (**B**) RTqPCR and Western blot analysis of the pluripotency gene NANOG in sphere-derived FACS-sorted CD133-positive and CD133-negative cells from 354 primary PDAC cells. RTqPCR data were normalized to β-actin and represented as fold change in compare to negative counterparts from at least three independent experiments; protein levels were normalized to β-actin.

Moreover, we additionally used autofluorescence as a marker to isolate and analyse cancer stem cells in our system. Autofluorescence in cells and tissues can be detected due to the presence of intrinsic biomolecules acting as endogenous fluorophores, with favourable spectral properties and quantum efficiency (excitation/emission ranges within the blue region where most endogenous fluorophores emit). Specifically, the autofluorescence in the cytoplasm of epithelial CSCs has a distinct green spectral profile (excitation and emission 480-490 nm and 530- 540 nm, respectively) and can be excited only with a standard blue laser, but not with yellow/green (e.g., 561 nm) or red (e.g., 640 nm) lasers. Autofluorescent cells have already been shown to possess cancer stem cells properties (Miranda-Lorenzo et al., 2014).

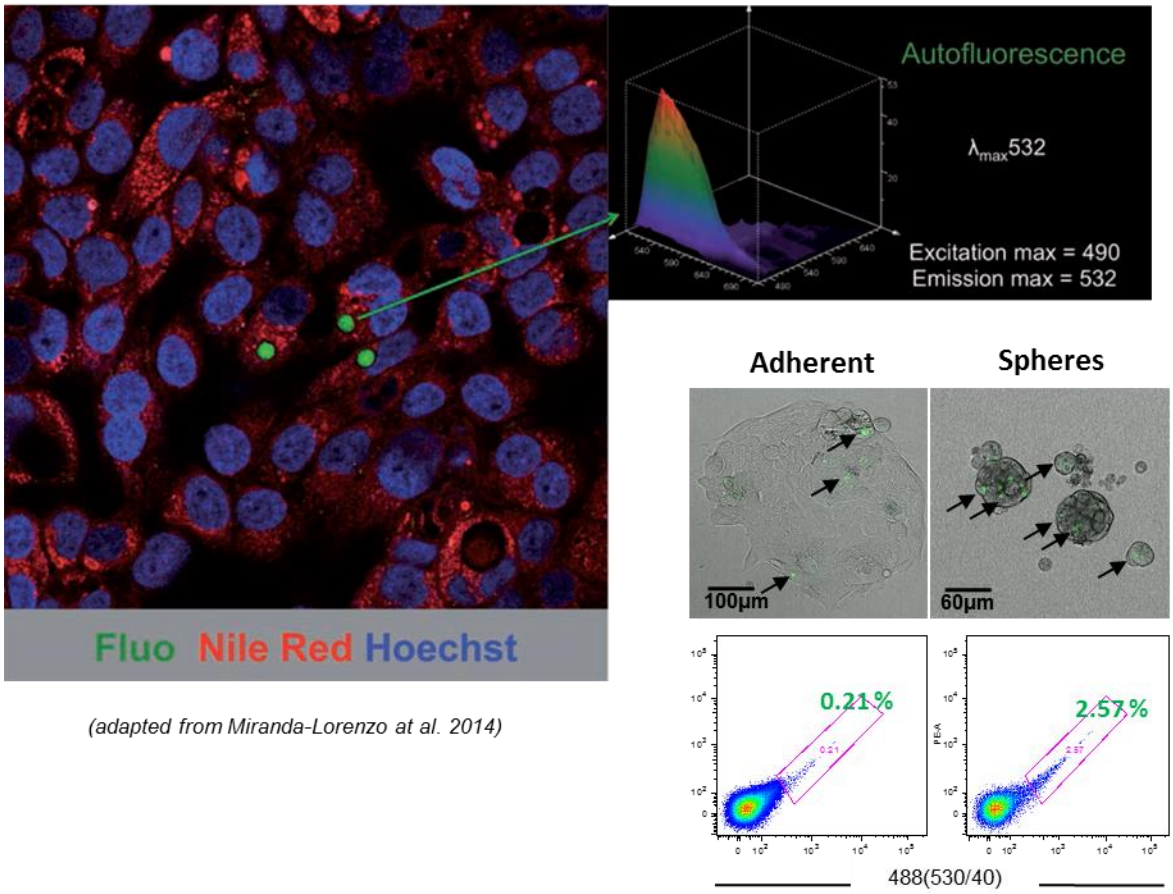

Figure 3. Autofluorescence in epithelial cancer stem cells. Confocal image of cancer cells derived from primary pancreatic cancer tissue showing a subset of cells with autofluorescent cytoplasmic vesicles (left). Spectral analysis of the autofluorescence demonstrated an emission maximum at 532 nm (upper right). Flow cytometry plots showing percentage of autofluorescence cells in adhered and sphere-derived primary PDAC 185 cells (right down). Figure is adapted from (Miranda-Lorenzo et al., 2014)

Following sorting of autofluorescent-positive and -negative cells from primary sphere-derived PDAC 185 cells we checked the expression of pluripotency-associated genes in autofluorescent-positive versus autofluorescent-negative cells and confirmed that autofluorescent-positive cells express significantly higher level of *NANOG*, *OCT3/4*, *SOX2* and *KLF4* genes compared to their autofluorescent-negative counterparts (Figure 4A). By Western blot analysis we confirmed our RTqPCR analyses and show that autofluorescent-positive cells express more Nanog protein versus autofluorescent-negative cells (Figure 4B).

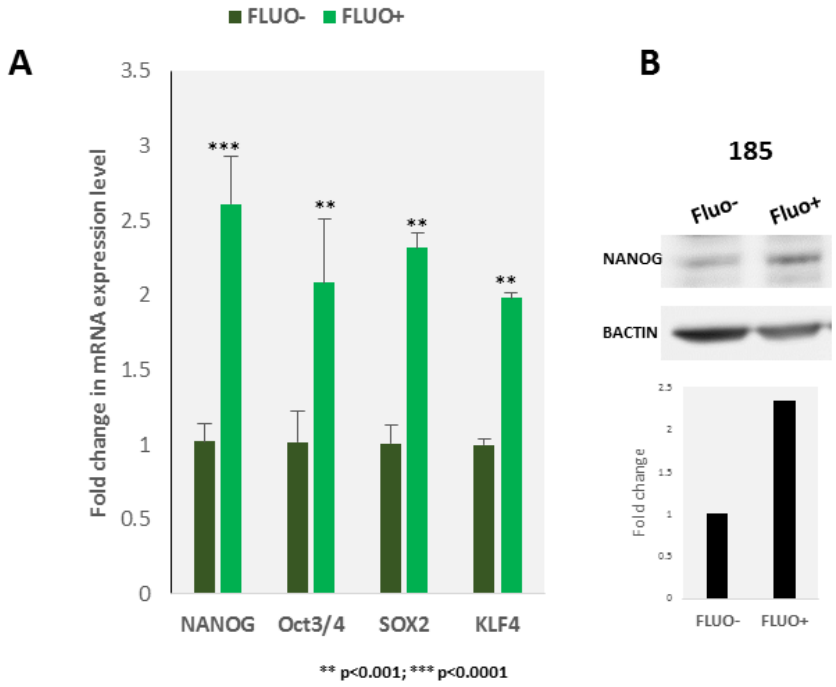

Figure 4. (**A**) RTqPCR analysis of pluripotency gene expression in FACS-sorted Autofluorescent-positive (Fluo+) and Autofluorescent-negative cells (Fluo-) from sphere-derived cells 185. Data are normalized to ß-actin and represented as fold change in compare to negative population (Fluo-) from at least three independent experiments (**B**) Western blot analysis of the pluripotency gene *NANOG* in FACS-sorted autofluorescent-positive and -negative sphere-derived 185 PDAC cells. Protein levels were normalized to β-actin and subsequently quantified by densitometry. Change in protein level are represented as fold change in compare to negative population (Fluo-).

1.2. Characterization of single cell derived tumours

It has long been known that cancer cells undergo clonal evolution in which genetic or epigenetic mutations occur stochastically in individual cells and are then subject to positive or negative selection depending on whether they confer a competitive advantage or disadvantage (Nowell, 1976). Thus this inherent cell heterogeneity should decrease in tumours generated from one single cell making further studies more specific to monoclonal population of cells. Epigenetics studies, for example could benefit from monoclonal system, allowing observation of differences that are due to non-genetic variations. For these reasons, we next set out to generate tumours from one single CSC. For this purpose we chose to inject autofluorescent-positive CSCs from FACS sorted adherent 185 PDAC cells subcutaneously into the flanks of nude mice, 12 months post injection, 2 out of 21 injections yielded tumours. At the histological level, the single cell-derived

tumours were histologically identical to the parental 185 xenograft from which the autofluorescent cells were derived. Single autofluorescent cell-derived tumours also recapitulated the heterogeneous composition of the original 185 xenograft tumour at the level of expression of the cell surface markers EPCAM and CD133 (data not shown). Importantly, we could also observe that single autofluorescent cell-derived tumours, in general, had a higher percentage of autofluorescent cells compared to the parental tumour (Figure 5).

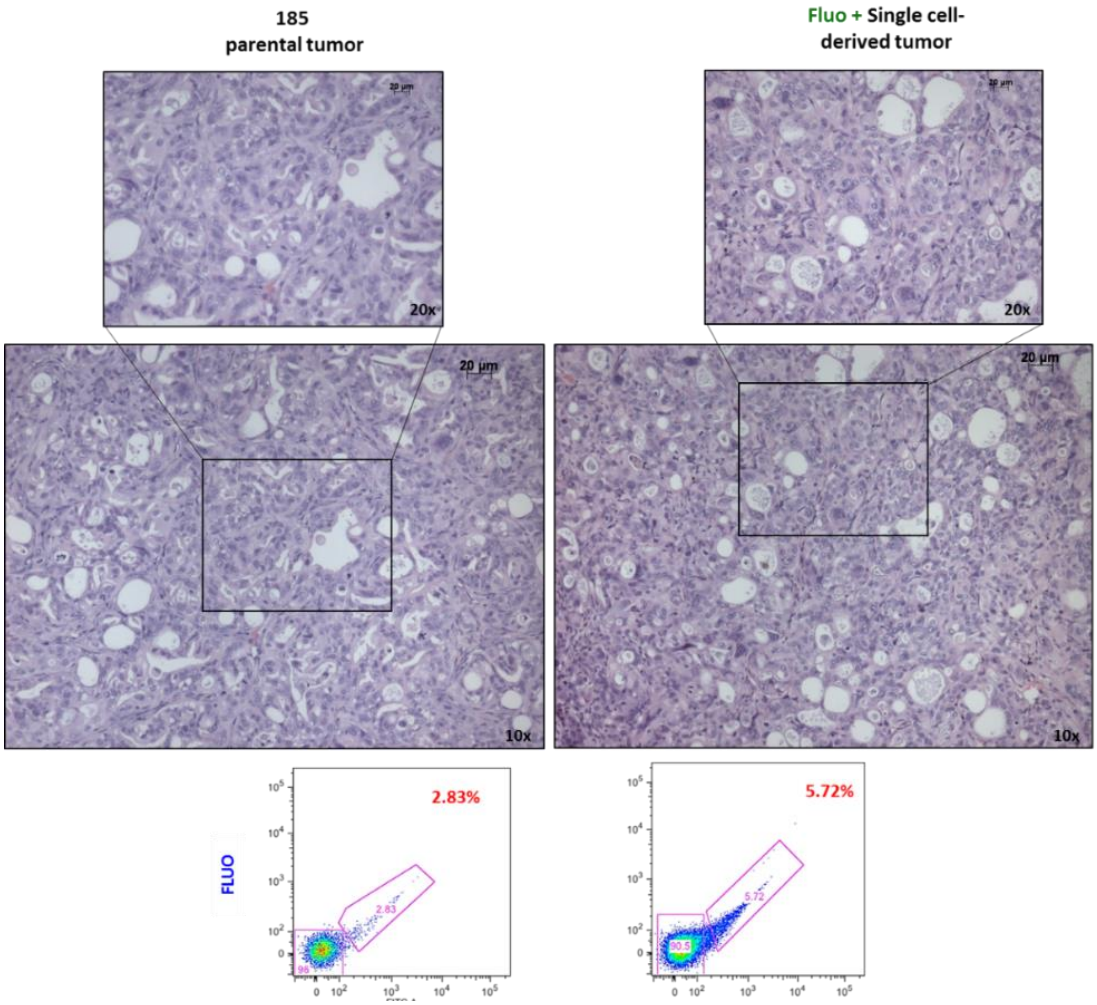

Figure 5. Representative pictures of H&E staining for PDAC 185 xenograft tumour and autofluorescent-positive single cell derived tumour (upper panel). Lower panel represent flow cytometry graphs showing percentage of autofluorscent cells in primary cultures from these tumours.

2. CHARACTERIZATION OF DNA METHYLATION LANDSCAPE IN PDAC CSCs

2.1. Methylation differences in NANOG gene between CSCs and non-CSC

Of the three methods used to identify and isolate CSCs, the autofluorescent phenotype reproducibly allowed for the isolation of CSCs that overexpressed pluripotency-associated factors at the mRNA and protein level. Based on these results, we used autofluorescence as a marker to separate cancer stem cells from non-stem cells in our subsequent studies. Since DNA methylation is very important mechanism in controlling the differential expression of pluripotency genes between normal stem cells and their more differentiated progeny (Meissner, 2010) we asked whether methylation could be responsible for the differential expression of pluripotency genes (e.g. *NANOG*) observed between autofluorescent-positive and autofluorescent-negative cells. We therefore focused on *NANOG* and selected two regions of the *NANOG* promoter for further analysis by bisulfite sequencing, a methodology commonly used to map DNA methylation because it provides a qualitative, quantitative and efficient approach to identify 5-methylcytosine at single base-pair resolution (Frommer et al., 1992). In total we covered 26 CpG sites of *NANOG* promoter including several that were already described to undergo changes in methylation status during reprograming of epithelial cells and generation of PDAC iPSC (Freberg et al., 2007; Kim et al., 2013). We found that on average the methylation of investigated regions were quite similar between autofluorescent-positive and -negative cells. *NANOG* Fragment 1 showed less than 50% methylation and *NANOG* Fragment 2 showed more than 90% of methylation (Figure 6A). When we looked at each CpG site individually, for *NANOG* Fragment 1 we observed that 6 out of 9 CpGs had lower level of methylation in autofluorescent-positive vs -negative populations of cells, suggesting that differential methylation at *NANOG* Fragment 1 may explain the differences observed in *NANOG* expression in autofluorescent positive versus autofluorescent negative cells. Methylation of single CpG dinucleotides were shown to be particularly important for transcriptional regulation of some other genes, such *MAGEA1, IL6* and *ZAP-70* (Claus et al., 2012;

Nile et al., 2008; Zhang et al., 2004). For *NANOG* Fragment 2 differences between autofluorescent-positive and autofluorescent-negative cells were less obvious (Figure 6B).

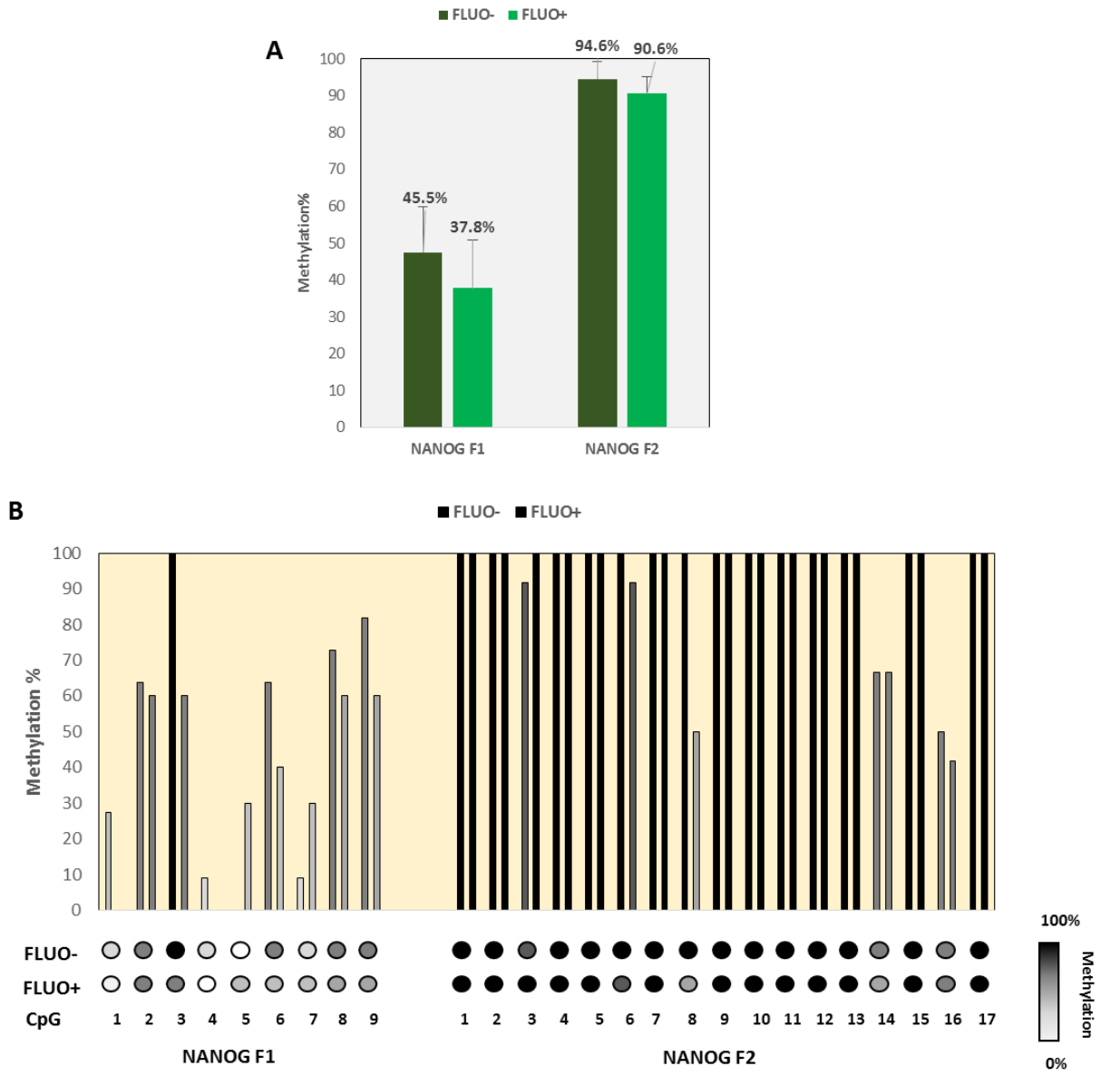

Figure 6. (**A**) PDAC 185 sphere-derived cells were FACS sorted for autofluorescence and subsequently DNA extracted from autofluorescent-positive (Fluo+) and -negative (Fluo-) cells was used for bisulfite conversion in order to assess DNA methylation of NANOG fragments. Graph is showing the average methylation level in each investigated fragment. **(B)** Representation of methylation percentage in individual CG site (26 in total) between autofluorescent-positive and -negative cells.

2.2. Identification of global methylation differences in CSCs vs non-CSCs using the 450K Illumina bead array

Since our bisulfite sequencing data suggested that CSCs may have a distinct methylation epigenome compared to non-CSCs, we performed genome-wide comprehensive methylation profiling using 450K Illumina bead array in order to gain insight into global DNA methylation differences between CSCs and their non-CSC counterparts. This platform allows for the comparison of the DNA methylation status of 485,578 CpG loci across samples, covering all RefSeq genes at single-nucleotide resolution, microRNAs and differently methylated regions (DMRs).

In order to observe greater differences and to assess issues of high heterogeneity and low polyclonality, we chose to use cells isolated from a primary pancreatic tumour, from liver metastases and from a single cell-derived tumour that was generated from CSCs isolated from the primary tumour. We reasoned that the CSC heterogeneity should be highest in the primary tumour, less in the metastatic tumour and homogenous in the CSCs isolated from the single cell-derived tumour. Thus, using primary pancreatic cancer cells (185), cells from liver metastasis (A6L), and cells obtained from a tumour generated from one single cell (185scd), we separated CSCs from non-CSCs using autofluorescence by FACS sorting (Figure 7A). We first evaluated the expression of pluripotency-associated genes to confirm that the autofluorescent-positive CSCs isolated from each tumour type for subsequent downstream array analyses expressed higher level of pluripotency genes, such as *NANOG*, *OCT3/4* and *SOX2*. In Figure 7B we show that indeed the autofluorescent-positive CSCs expressed higher levels of the pluripotency-associated genes analysed compared to the autofluorescent-negative cells isolated in parallel.

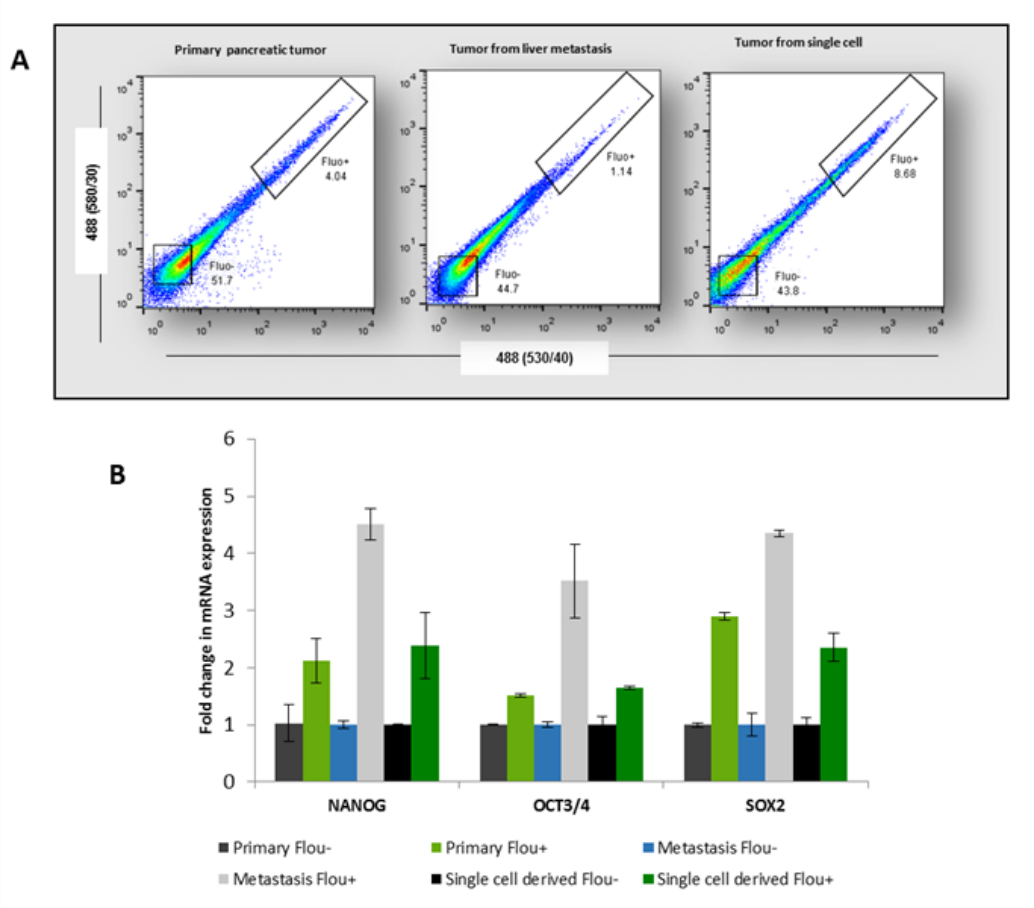

Figure 7. (**A**) Flow cytometry analysis of autofluorescent content in sphere-derived cells (185, A6L and 185 derived from single cell tumour) (**B**) RTqPCR analysis for pluripotency-associated genes in FACS sorted autofluorescent-positive (Fluo+) and -negative (Fluo-) cells. Data are normalized to ß-actin and represented as fold change in compare to negative population (Fluo-).

The main goal of DNA methylation mapping is the identification of systematic differences between groups of samples. Before analysing the CpG methylation data we followed the pipeline illustrated in Figure 8. Following a normalization step we excluded probes with low detection p value and to avoid gender specific bias we excluded probes found on sex chromosomes. Moreover since it was shown in a recent publication (Chen et al., 2013) that some autosomal probes can cross-hybridize to the sex chromosomes, we excluded those probes as well. Additional probes that were removed from our data were SNP, as DNA methylation measured at probes with SNPs in the target site may be compromised by sample genotype (Fraser et al., 2012).

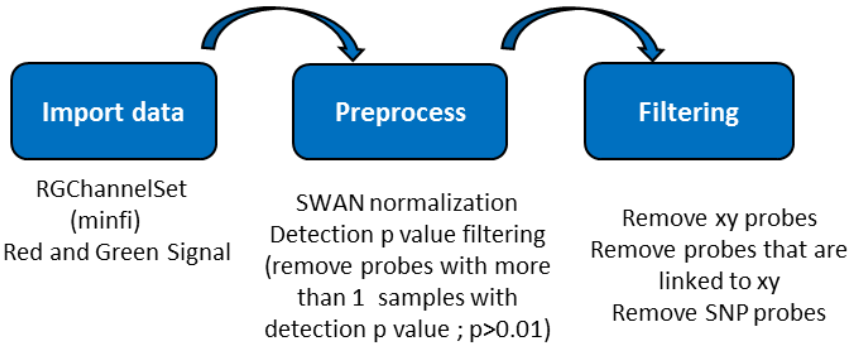

Figure 8. Workflow for DNA methylation analysis

We performed principal component analysis to see how samples segregate based on their normalized β methylation values (Figure 9). Our analysis showed that autofluorescent-positive cells were closer to autofluorescent-negative cells from each sample, meaning that the difference in methylation profiles were greater between different tumour entities than between two cell populations.

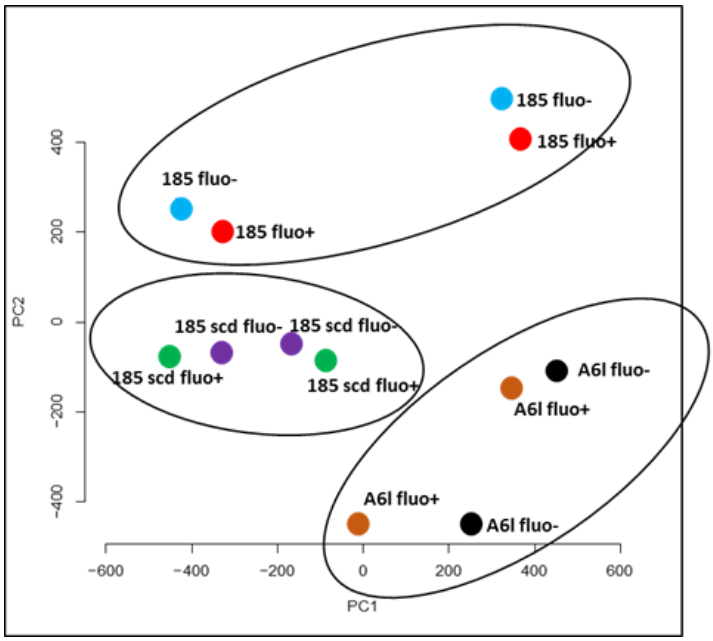

Figure 9. Principal component analysis (PCA) of autofluorescent-positive (Fluo+) and –negative (Fluo-) cells from different primary PDAC sphere-derived cells based on their normalized β-values. PCA shows three distinct groups, upper group of autofluorescent-positive and -negative from two biological duplicate of cells obtained from primary tumour (185), middle group of autofluorescent-positive and -negative cells from two biological duplicate of cells from one single cell-derived tumour (185scd) and lower group of autofluorescent-positive and -negative cells from liver metastasis (A6L).

Next we wanted to see if autofluorescent-positive and -negative cells were different in their global methylation level. We compared these two populations of cells individually from different tumours and observed a slight but significant increase in global methylation in autofluorescent-positive compared to autofluorescent-negative cells (Figure 10).

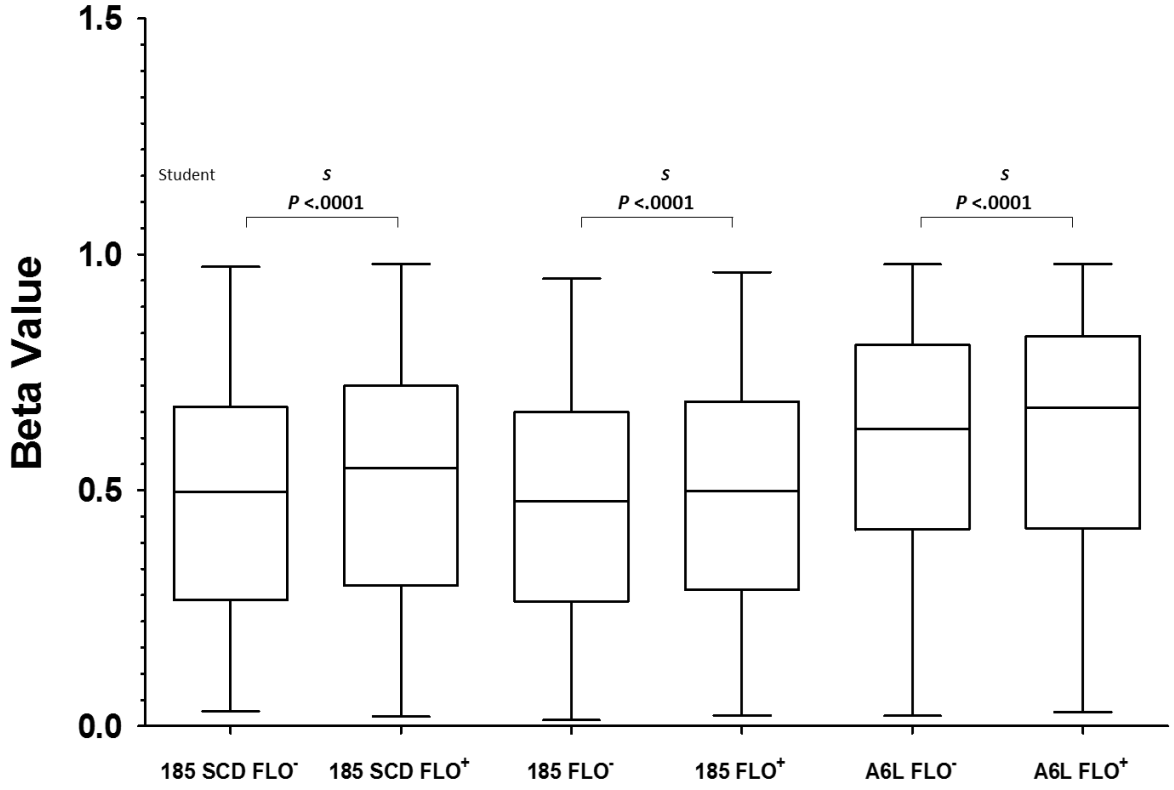

Figure 10. Box plot representing global methylation levels in representative pairs of autofluorescent-positive and autofluorescent-negative cells from different primary PDAC sphere-derived cells.

After an initial analysis of global trends in a DNA methylation data set, we next aimed to identify differentially methylated probes that exhibit consistently different DNA methylation levels between sample groups.

2.3. Identification of differently methylated probes

Different tumour samples that we used for methylation analysis allow us to ask various questions regarding CSC biology. As mentioned, we reasoned that the CSCs heterogeneity should be highest in the primary tumour, less in the metastatic tumour and homogenous in the CSCs isolated from the single cell-derived tumour. After observing that methylation differences are greater between different tumours than between autofluorescent-positive and -negative population according to PCA (Figure 9), we asked further 1) how different in methylation signature are autofluorescent-positive and -negative population of cells in each tumour separately 2) can we find common probes differently methylated and if this overlap is greater with decrease in cancer cell heterogeneity 3) if we neglect heterogeneity and polyclonality, and compare autofluorescent-positive from all tumours to autofluorescent-negative cells, what common differently methylated probes will we be able to discover?

Firstly, we looked for differently methylated probes between autofluorescent-positive and autofluorescent-negative population of cells in each tumour separately. We initially defined a probe as unmethylated if β-value was lower than 0.3 in both autofluorescent-positive and -negative cells. Similarly, we defined a probe as methylated if β-value was higher than 0.7 in both autofluorescent-positive and -negative cells. As only two biological replicate of autofluorescent-positive and autofluorescent-negative cells from each tumour were available, we used next criteria to define hyper- and hypomethylated probes. A probe was classified as hypermethylated in autofluorescent-positive population if β-value was higher than 0.7 in autofluorescent-positive and lower than 0.7 in autofluorescent-negative (same criteria was used to define hypermethylated probes in autofluorescent-negative population). Conversely, the probe was defined as hypomethylated in autofluorescent-positive population if β-value was lower than 0.3 in autofluorescent-positive and higher than 0.3 in autofluorescent-negative (same criteria was used to define hypomethylated probes in autofluorescent-negative population) (Figure 11A). Using this approach we found that both autofluorescent-positive and -negative cells displayed various number of differently methylated probes with (Figure 11B).1) Autofluorescent-positive population of cells across all tumours having more hypermethylated probes than autofluorescent-negative cells and 2) that cells from liver metastasis showed highest number of differently methylated probes. Higher

number of hypermethylated probes in autofluorescent-positive population of cells could be explained by their significantly higher global methylation level in compare to autofluorescent-negative counterparts, as shown in Figure 10.

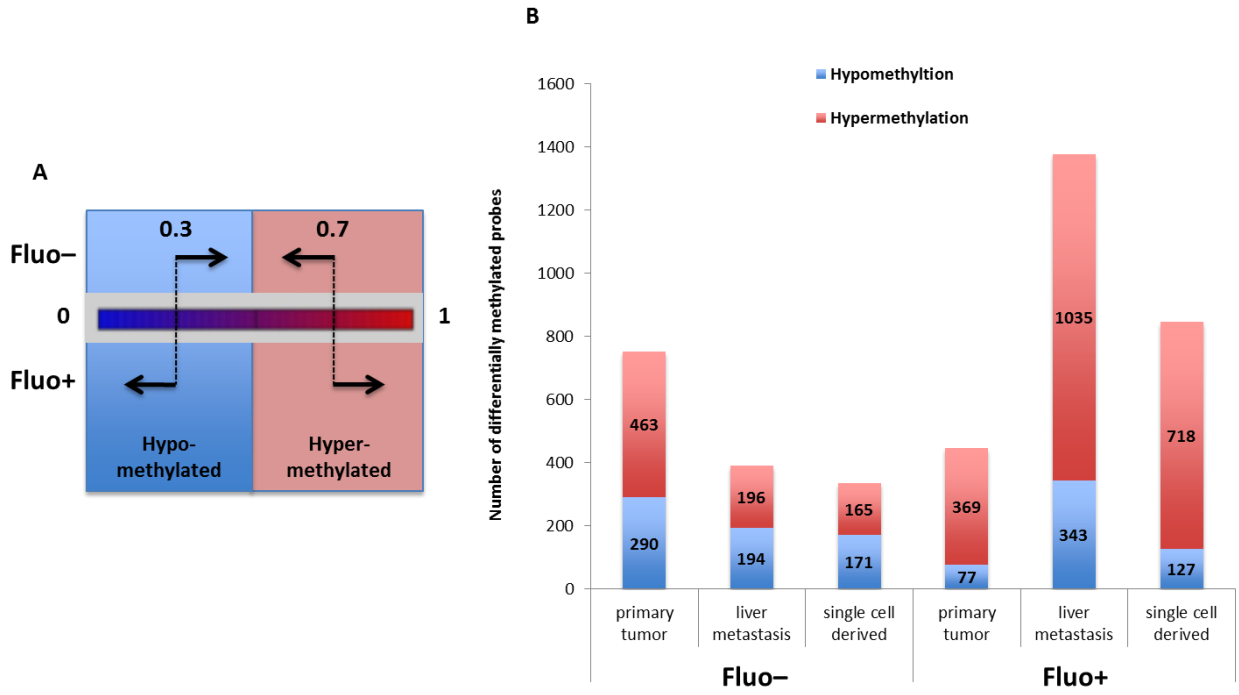

Figure 11. (A) Box graph showing our strategy to identify differently methylated probes in autofluorescent-positive (Fluo+) and -negative (Fluo-) cells in each tumour individually (185, A6L and 185scd). **(B)** Graphs showing the number of hyper and hypomethylated probes that we identified in autofluorescent-positive and -negative cells from primary tumour (185), liver metastasis (A6L) and single cell derived tumour (185scd).

Moreover we were interested to find common differently methylated probes between tumours and if this overlap is greater with decrease in cancer cell heterogeneity (i.e, between liver metastasis and single cell-derived tumours, in comparison to other overlaps). So we looked into probes that are common between primary tumour and liver metastatic tumour and we found genes that were involved in invasion and migration such as *TGFBR3* or genes involved in stem cell maintenance *MSI2*. When we looked into probes that were common differently methylated in primary tumour and single cell-derived tumour we found that this probes were related to genes involved in differentiation *BDNF*, *UACA* and lipid metabolism *OSBPL9* and *IDH2*. Interestingly when we look into probes that were hypo- or hypermethylated and common between liver metastasis and single cell-derived tumour we found panel of genes involved in processes very

important for cancer cells such as invasion, migration, and metastasis like *TBCA*, *PARD3*, *MAGI2*, *ROBO2*, *SNAI1*, and *VMP1*. Also some of these genes were involved in maintenance of stem cells like *CAMK4* or Wnt and Notch signalling like *NOTCH4* and *MAML3* (Figure 12).

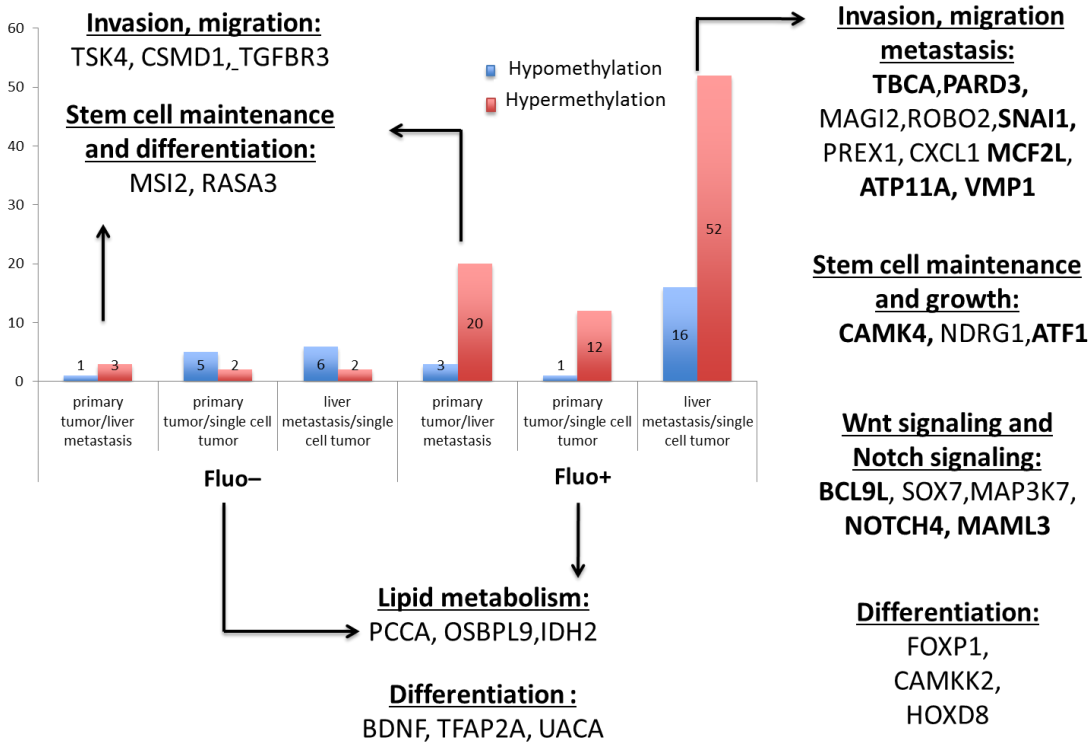

Figure 12. We looked for common differently methylated probes between tumours- primary tumour vs. liver metastasis, primary tumour vs. single cells-derived tumour and liver metastasis vs. single cell-derived tumour. We show that genes common between each groups are related to processes involved in invasion, migration, stem cell maintenance or Notch signalling.

Interestingly, we did observe greater number of common differently methylated probes between liver metastasis and single cells-derived tumour. Moreover, many of common genes were involved in processes related to CSCs, such as invasion, metastasis and Notch signalling. So we proceeded in checking the expression of these genes in order to see if they show difference in mRNA expression level between autofluorescent-positive and -negative cells. We FACS sorted autofluorescent-positive and -negative cells from independent set of sphere-derived cells from liver metastasis (A6L) and single cell-derived tumour cells (185scd) and we observed that selected genes, like *MCF2L*, *VMP1*, and *MAML3* were more expressed in autofluorescent-positive than in autofluorescent-negative in both cells tested (Figure 13). This was not the case for SNAI1 that showed no difference between autofluorescent-positive and -negative cells from both tumours or

CAMK4 and *NOTCH4* whose expression were decreased only in autofluorescent-positive cells from single cell-derived tumour cells (Figure 13).

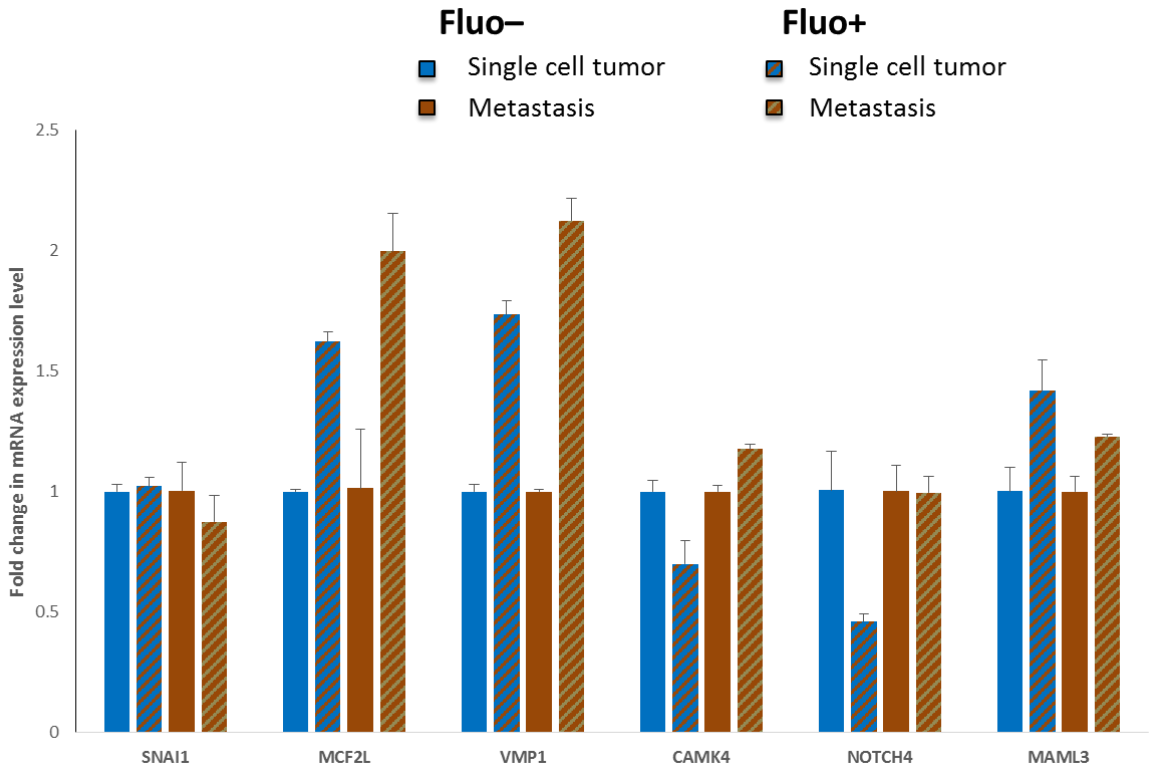

Figure 13. RTqPCR analysis of selected target genes in independent set of autofluorescent-positive (Fluo+) and autofluorescent-negative (Fluo-) cells FACS sorted from sphere-derived cells from liver metastasis and single cell-derived tumour. Data were normalized to β-actin and represented as fold change in compare to negative population (Fluo-).

Furthermore, many of the genes that we selected as common differently methylated between single cell-derived tumour and liver metastasis, and subsequently showed higher expression in autofluorescent-positive cells in both tumours (*MCF2L* or *VMP1*- Figure 13), were not differently expressed between autofluorescent-positive and -negative cells from primary tumour (data not shown). This difference in gene expression between population of autofluorescent-positive and autofluorescent-negative cells from different tumours could be explained by the decrease in gene expression noise, in more homogenous population of cells, such as one present in monoclonal and metastatic tumour. However, some target genes showed similar pattern of expression between autofluorescent-positive and -negative cells across all tumour. One of such targets was *MAML3*.

Mastermind (Mam) is one of the elements involved in Notch signalling, a system that plays a pivotal role in cell-cell communication, embryogenesis, cellular differentiation and stem cell renewal. Notch signalling has also been shown to be important in PDAC (Miyamoto et al., 2003). Thus, we decided to investigate its role in PDAC CSCs due to its important role in Notch signalling. We show that *MAML3* is significantly more expressed in autofluorescent-positive compared to autofluorescent-negative cells from primary 185 tumour. Moreover, we saw a similar up regulation of *MAML3* mRNA expression when we compared sphere-derived cells to adherent cells (Figure 14).

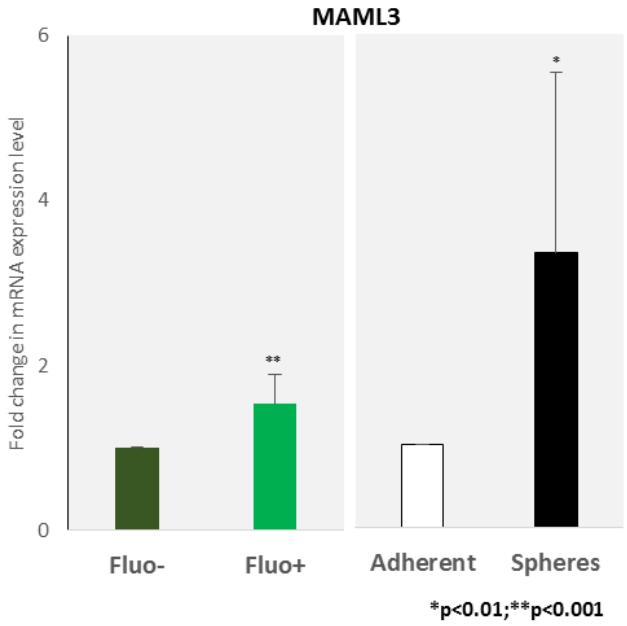

Figure 14. RTqPCR analysis of MAML3 gene expression in FACS sorted autofluorescent-positive (Fluo+) versus autofluorescent-negative (Fluo-) sphere-derived primary 185 tumour cells (left) and sphere-derived cells versus adherent cells (right).

Importantly, *MAML3* was identified as being hypermethylated in our methylation data analysis, however, its mRNA expression was higher in CSCs (autofluorescent-positive and sphere-derived cells) versus non-CSCs (autofluorescent-negative and adherent cells). Thus, this result indicated that the DNA methylation status of the *MAML3* gene does not correlate with its gene expression. Moreover, the up regulation of *MAML3* indicate activation of the Notch pathway in autofluorescent-positive cells. These data promoted us to design shRNAs targeting *MAML3* to assess its role in PDAC CSC biology (Figure 15). Unfortunately all attempts to date to silence this

gene using an inducible lentiviral-knockdown approach were unsuccessful. Thus, the question of whether *MAML3* plays important role in CSCs remains to be answered.

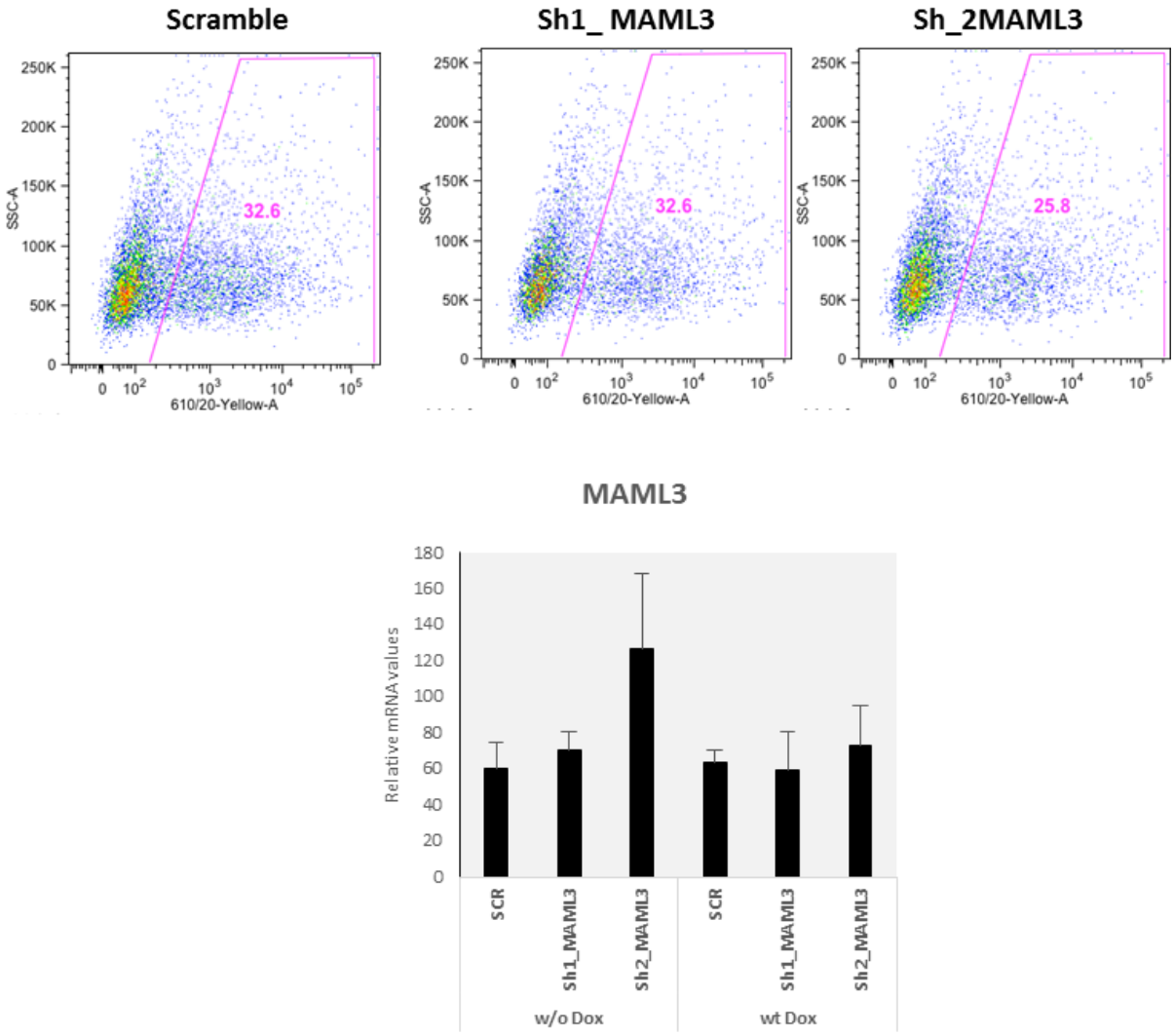

Figure 15. FACS plots representing sorting strategy used for isolation of cells expressing inducible lentiviral constructs containing shRNAs directed against *MAML3* gene (upper panels) and RTqPCR analysis of *MAML3* in cells expressing a scrambled or two different *MAML3* shRNAs 72h post doxycicline induction (lower panel) . Data were normalized to b-actin and represented as relative mRNA levels.

The fact that we had available only two biological replicates of Autofluorescent-positive and -negative cells from various different tumour origins, limited possibility of performing statistical analysis of methylation data. For this reason we next decided to use another approach to identify differently methylated probes between autofluorescent-positive and -negative cells. We pared all autofluorescent-positive cells from different tumours and compared them to all

autofluorescent-negative cells. This approach allowed us to identify 9,230 probes that were differential methylated, of which 7,848 were more methylated in Fluo + cells (hypermethylated), and 1,382 were less methylated in autofluorescent-positive (hypomethylated) with adjusted p-values of less than 0.05 (Figure 16).

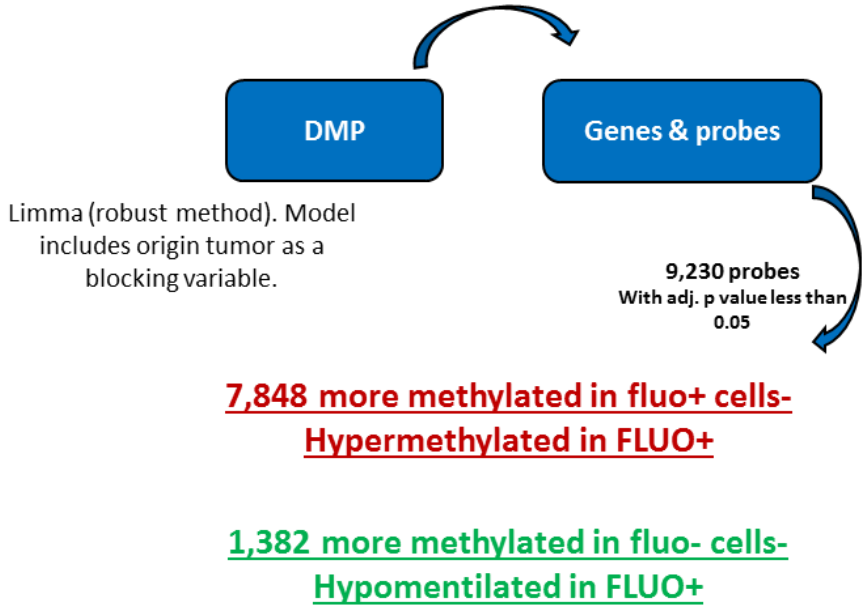

Figure 16. Workflow for identification of differently methylated probes using the Limma analysis method.

One observation from our first approach was that many genes that we found as interesting targets, were the once that had differently methylated probes located in gene regions outside of CpG island, like for example in gene body regions. Studies showed that gene body methylation is actually positively correlated with gene expression (Kulis et al., 2012; Maunakea et al., 2010; Varley et al., 2013), and genes such as one from our list for which we did not observed correlation in methylation and gene expression, *MAML3* was hypermethylated in probes found in the body of the gene. However, even though the relationship between DNA methylation and gene expression is more complicated than previously thought and not always directly related to methylation in CpG islands, many studies still report positive correlations between gene expression and CpG island methylation. Moreover, genes that are hypermethylated in their promoters and silent, could be very attractive targets for epigenetically targeted therapy using demethylation drugs.

Thus, we decided to select from our list of hyper and hypomethylated probes, only probes found in CpG islands and promoters and we were able to reduce our list of "candidate" genes to 908 hypermethylated genes and 386 hypomethylated genes. Additionally, we used a second filtering approach by selecting genes with fold change difference higher than 1.3 in the hypermethylated group and less than 0.76 in the hypomethylated group to further narrow our list of interesting targets. This approach yielded a list of 546 and 213 genes that were hypermethylated and hypomethylated, respectively (Figure 17).

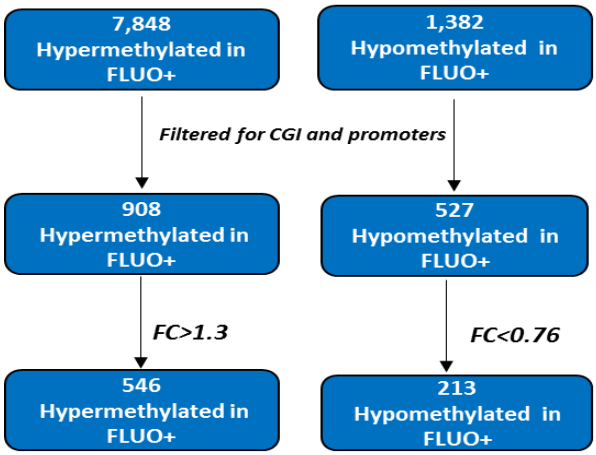

Figure 17. Workflow for data filtering

We first looked at hypermethylated group of genes as these genes could potentially be targets for reactivation using epigenetic therapy. We analysed the list of hypermethylated genes using IPA (Ingenuity Pathway) and discovered interesting top pathways represented in our dataset (Figure 18), such as pathways related to the Polo-like kinase. Moreover, we also found pathways involved in DNA damage repair, TGF-ß signalling and epithelial to mesenchymal transition (EMT) pathway regulation.

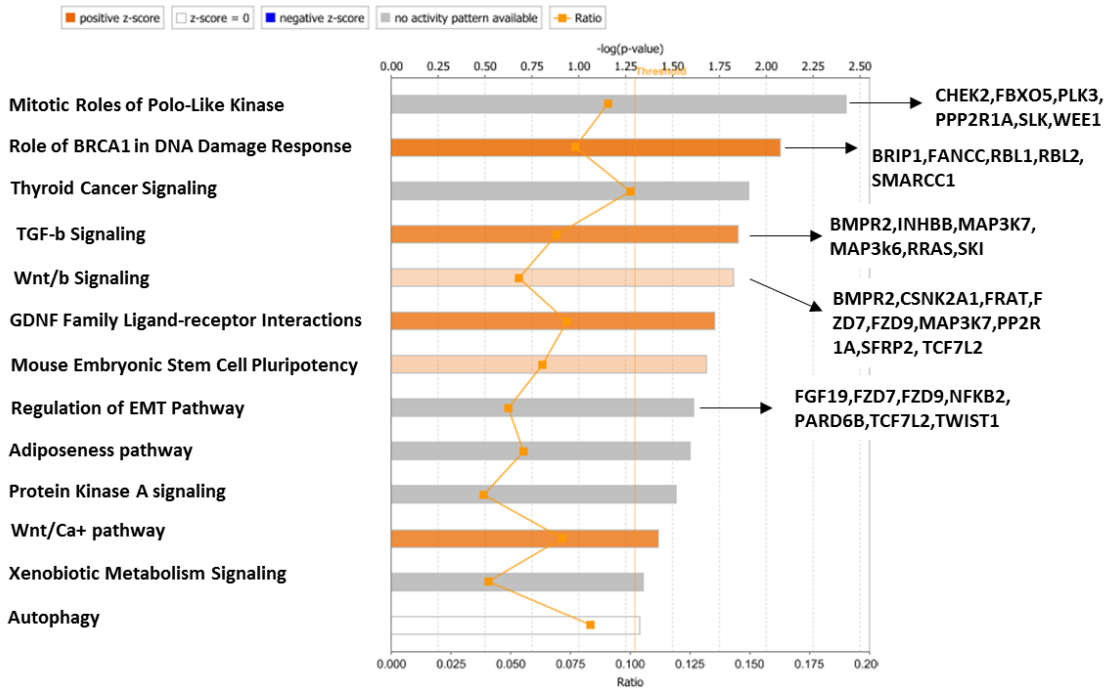

Figure 18. Ingenuity pathway analysis on hypermethylated genes in autofluorescent-positive CSCs from all tumours analyzed

One of the top pathways represented in our dataset was pathway related to Polo-like kinase. Polo-like kinases (Plks) represent a family of highly conserved serine–threonine kinases that play essential roles in cell cycle progression and in the cellular response to different types of stress, such as genotoxic stress. Among genes that belong to this pathway and that were hypermethylated in autofluorescent-positive cells was Polo-like 3 kinase (Plk3). Interestingly, the expression of Plk3 was found to be reduced in certain types of human cancer like head and neck, lung and liver (Li et al., 1996). Moreover, it was found to be silent by promoter hypermethylation 37.3% of HCC, and this was inversely correlated with the survival of HCC patients (Dai et al., 2000). The role of Plk3 kinase in pancreatic cancer is not very well investigated, even though some reports suggest that could also function as a tumour suppressor and that loss of Plk3 could be important for pancreatic cancer progression (Li, 2012). Thus, for the future direction it would be worth investigating the role of this kinase in pancreatic CSCs.

3. TARGETING DNA METHYLATION IN PANCREATIC CANCER STEM CELLS

3.1. Higher DNMT expression is characteristic of PDAC cancer stem cells

Although our global methylation array data fell short of providing us with 1) a black and white picture of the methylation status of CSCs versus non-CSCs and 2) efficiently validatable list of target genes shown to be necessary for CSC biology, our studies did, however, highlight that CSCs (i.e autofluorescent-positive cells) have a higher level of global methylation compared to their negative counterparts, regardless of the heterogeneity or polyclonality of the CSC populations present in the tumours analysed (Figure 10). Consequently, we next asked if this general hypermethylation could be explained by differential expression of DNA methyltransferase proteins, which are critical for catalysing the transfer of a methyl group to DNA (Beard et al., 1995; Li et al., 1992). Therefore we first looked at the mRNA expression of *DNMT1*, *DNMT3a* and *DNMT3b* in autofluorescent-positive versus autofluorescent-negative cells by RTqPCR analysis and observed that two of the DNMTs, specifically *DNMT1* and *DNMT3a* were significantly overexpressed in autofluorescent-positive cells compared to autofluorescent-negative cells. This observation, however, could not be confirmed at the level of protein for *DNMT1* (Figure 19).

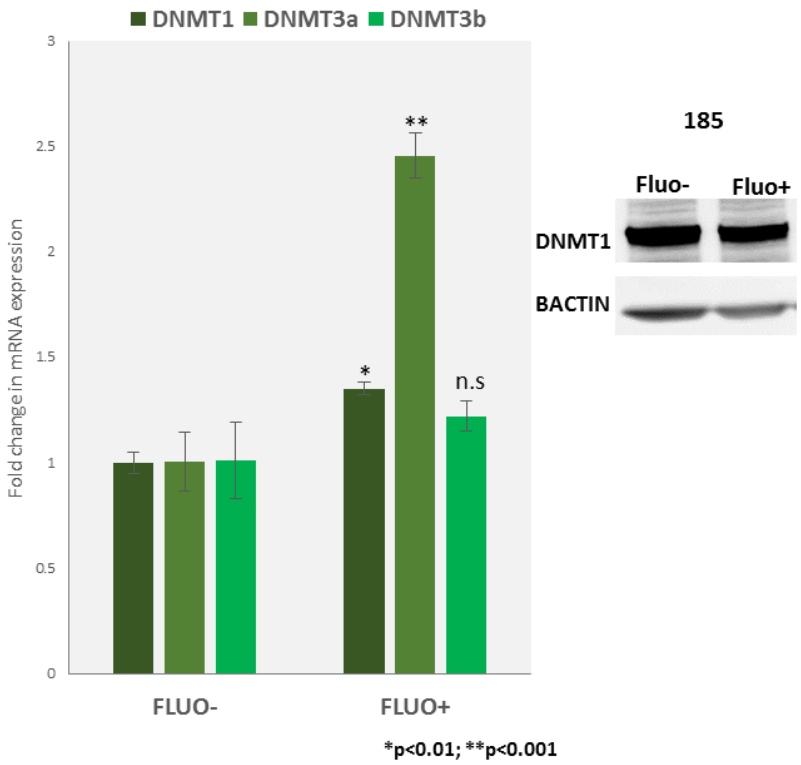

Figure 19. RTqPCR analysis of three DNMT genes, *DNMT1*, *DNMT3a* and *DNMT3b* in FACS sorted 185 sphere-derived autofluorescent-positive (Fluo+) and –negative (Fluo-) cells. Data are normalized to β-actin and represented as fold change in compare to autofluorescent-negative population (left); western blot analysis of DNMT1 protein level in FACS sorted 185 sphere-derived autofluorescent-positive and -negative cells (right). Protein level were normalized to β-actin.

Moreover, we checked the mRNA expression of *DNMT1*, *DNMT3a* and *DNMT3b* in adherent and sphere cultures, and we found that *DNMT1* was consistently up regulated across different primary sphere-derived cells versus adherent cells (Figure 20A). Protein western blot analysis of DNMT1 expression showed higher expression in spheres, confirming the results obtained at the gene expression level (Figure 20B).

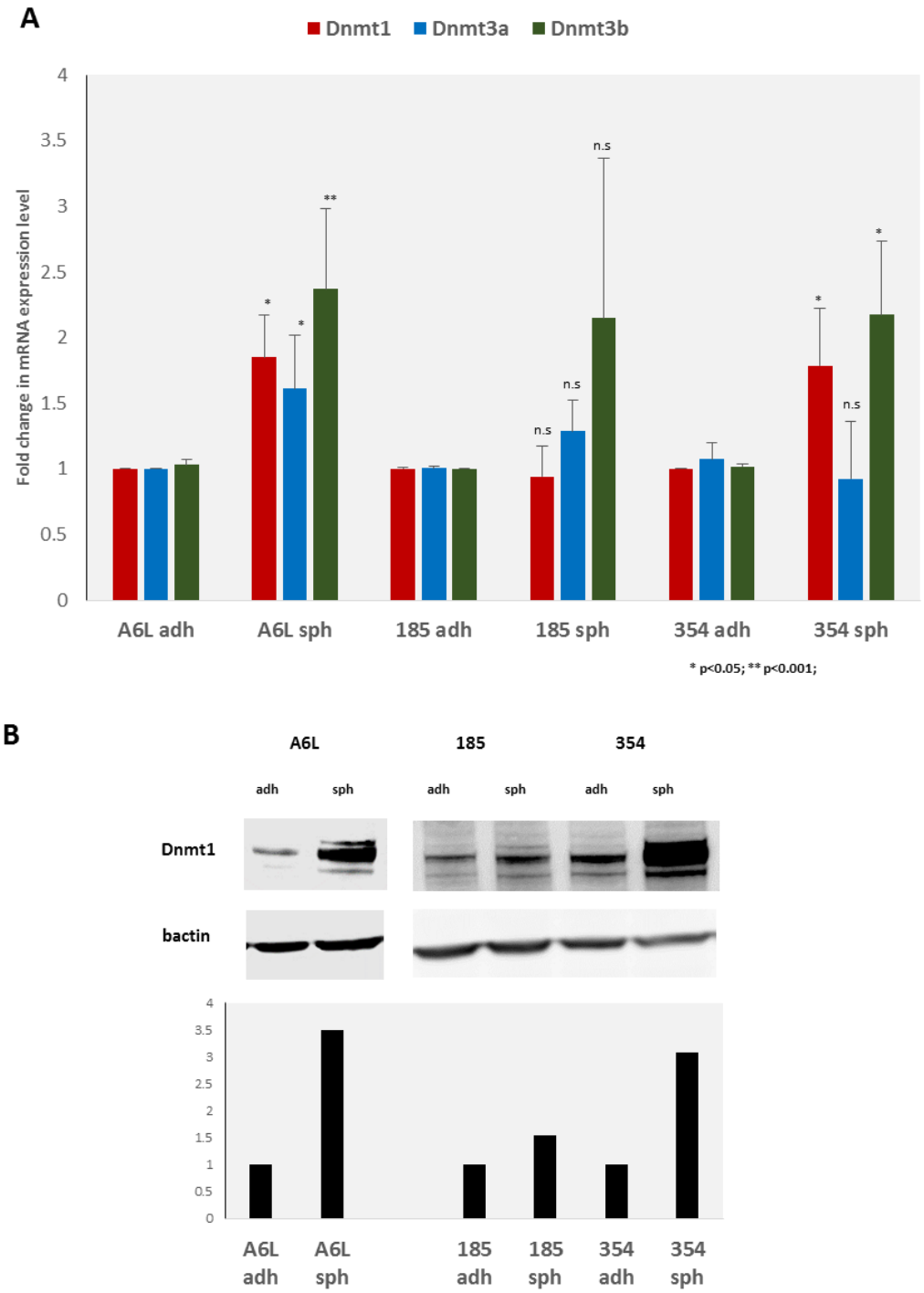

Figure 20. (**A**) RTqPCR analysis of three DNMT genes, DNMT1, DNMT3a and DNMT3b in primary PDAC adherent (adh) and 7 days old sphere (sph) cells (A6L, 185 and 354). Data are normalized to β-actin and represented as fold change in compare to adherent cells. (**B**) Western blot analysis of DNMT1 protein across a panel of different primary sphere-derived cells versus adherent cells (A6L, 185 and 354). Protein levels are normalized to β-actin and subsequently quantified by densitometry. Change in protein levels are represented as fold change in compare to adherent cells.

The aforementioned experiments indicated that cancer stem cells express higher level of DNMT methylentrasferase proteins, with DNMT1 showing the most consistent up regulation. This is in line with previous studies that have also shown that DNMTs, are not only more expressed in cancers compared to normal tissue, like colon cancer (el-Deiry et al., 1991), prostate cancer (Patra et al., 2002), breast cancer (Girault et al., 2003), liver cancer (Oh et al., 2007) and in leukaemia (Melki et al., 1998), but also that DNMT1 has been shown to be essential for the maintenance of leukaemia stem cells (Broske et al., 2009), skin progenitor cells (Sen et al., 2010) and more recently, studies have demonstrated that DNMT1 functions in the maintenance of human colon CSCs (Morita et al., 2013). The sum of these data and observations makes this protein a very attractive target for chemotherapy and chemoprevention. In order to test the biological role and relevance of DNMT1 in the context of pancreatic CSCs, we performed pharmacological targeting-based experiments using the DNA methylation inhibitor Zebularine.

3.2. DNMT inhibitor Zebularine effectively decreases DNMT1 protein levels

Zebularine is a cytidine analog that was originally developed as an inhibitor for cytidine deaminase. It acts primarily as a trap for DNMT proteins by forming tight covalent complexes between DNMT protein and zebularine substitute DNA (Cheng et al., 2003). In order to investigate the cytotoxic effect of zebularine on different primary pancreatic cell cultures we first performed a cytoxicity Toxilight assay, exposing cells to different concentrations of zebularine for 24h and subsequently measuring the effect of this compound on cell viability to determine a non-toxic dose for further studies. Since concentration higher than 100μM were considered to be toxic, we chose to use zebularine at a concentration of 75μM for the following studies (Figure 21).

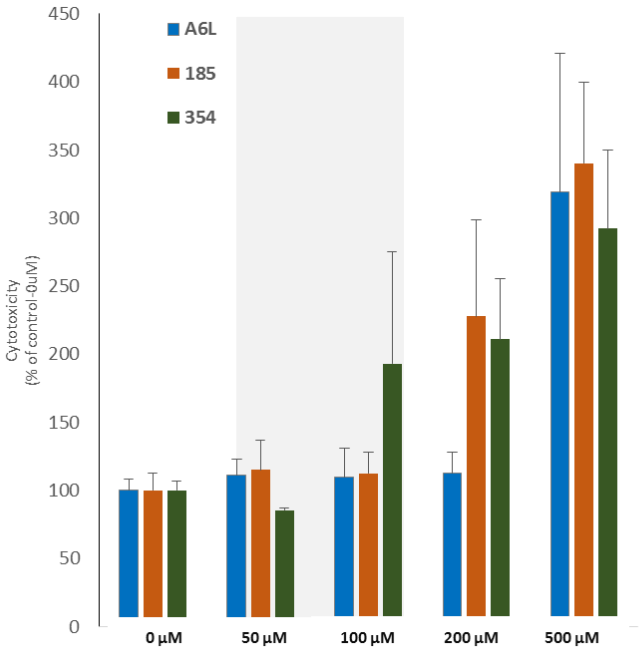

Figure 21. Graph showing toxicity following 24h treatment with different concentrations of Zebularine. Primary PDAC cells (A6L, 185 and 354) were plated as adherent monolayers in 96 well plates and 24h later treated with increasing concentrations of Zebularine (0 to 500µM). Twenty four hours later, cellular cytotoxicity was evaluated. Analysis were performed in triplicate and results are represented as percentage of cytotoxicity compared to control samples (0µM). 75µM concentration was chosen for future studies.

In order to test the effect of zebularine in the context of pancreatic cancer stem cells, we treated sphere-derived cells with zebularine at a concentration of 75µM. We observed that treatment was able to decrease DNMT1 protein level in three primary pancreatic sphere-derived PDAC cultures (Figure 22A). At the gene level we observed no difference in DNMT1 mRNA levels upon zebularine treatment except in 185 cells, confirming previous reports (Cheng et al., 2004) (Figure 22B).

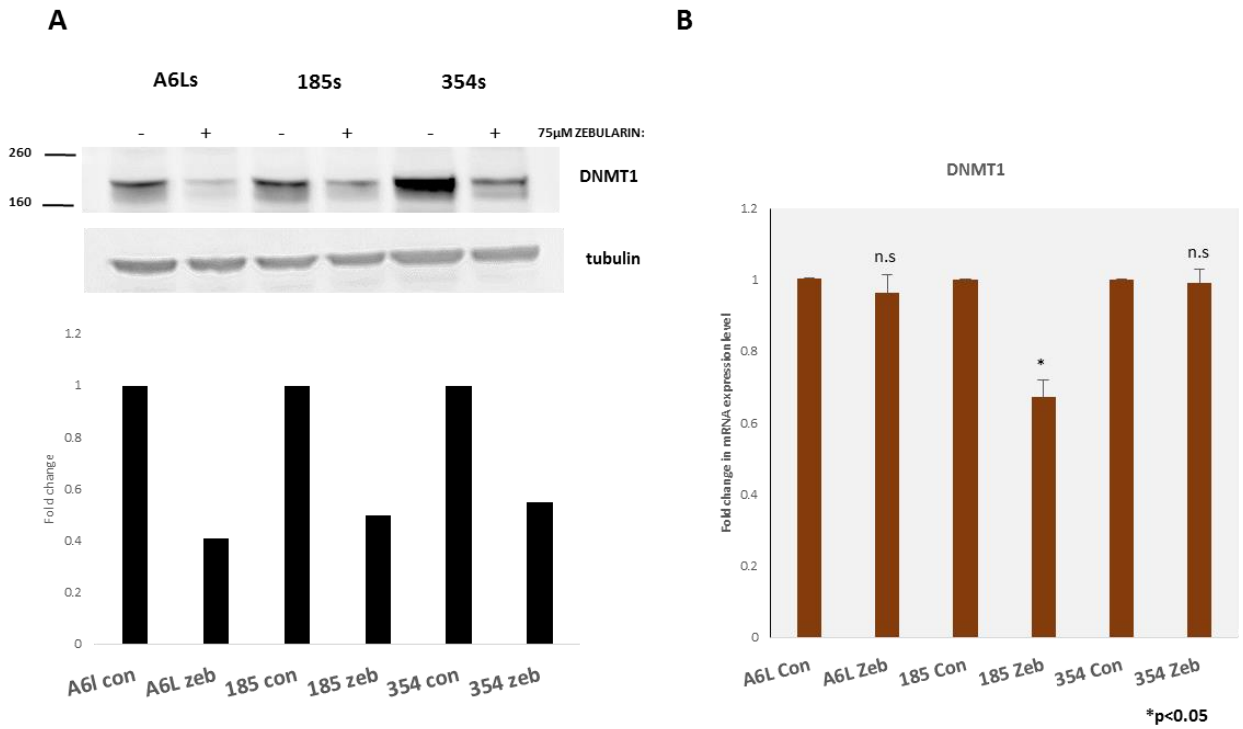

Figure 22. (**A**) Western blot analysis of DNMT1 protein in various primary PDAC sphere-derived cells (A6L, 185 and 354) untreated and treated with Zebularine. Cells were plated in sphere culture conditions and treated with 75μM of Zebularine. Drug was administrated every 2nd day directly into culture media for 7 days, when spheres were filtered through 50μm filters and lysed for protein extraction. Protein levels were normalized to tubulin and subsequently quantified by densitometry. Change in protein levels are represented as fold change in compare to untreated cells. (**B**) RTqPCR analysis of DNMT1 gene expression in various primary PDAC sphere-derived cells (A6L, 185 and 354) after treatment with Zebularine. Drug was added every 2nd day directly into culture media for 7 days, after which spheres were filtered and lysed for RNA extraction. Data are normalized to β-actin and represented as fold change in compare to untreated cells.

3.3. Cancer stem cell phenotype

In order to test if the observed decrease in DNMT1 protein levels had a biological impact on pancreatic CSCs, we measured the percentage of CSCs following Zebularine treatment by measuring the percentage of autofluorescent or CD133-positive cells by flow cytometry. Treatment of sphere-derived cells with the DNMT inhibitor zebularine marginally decreased the percentage of autofluorescent-positive cells from two different PDAC tumours A6L and 185 (Figure 23A). Regarding the expression of CD133, however, we observed a significant decrease in the percentage of CD133-positive cells and a corresponding increase in CD133-negative cells

following treatment (Figure 23B). The apparent decrease in *CD133* was confirmed at the mRNA level by RTqPCR analysis (Figure 23C).

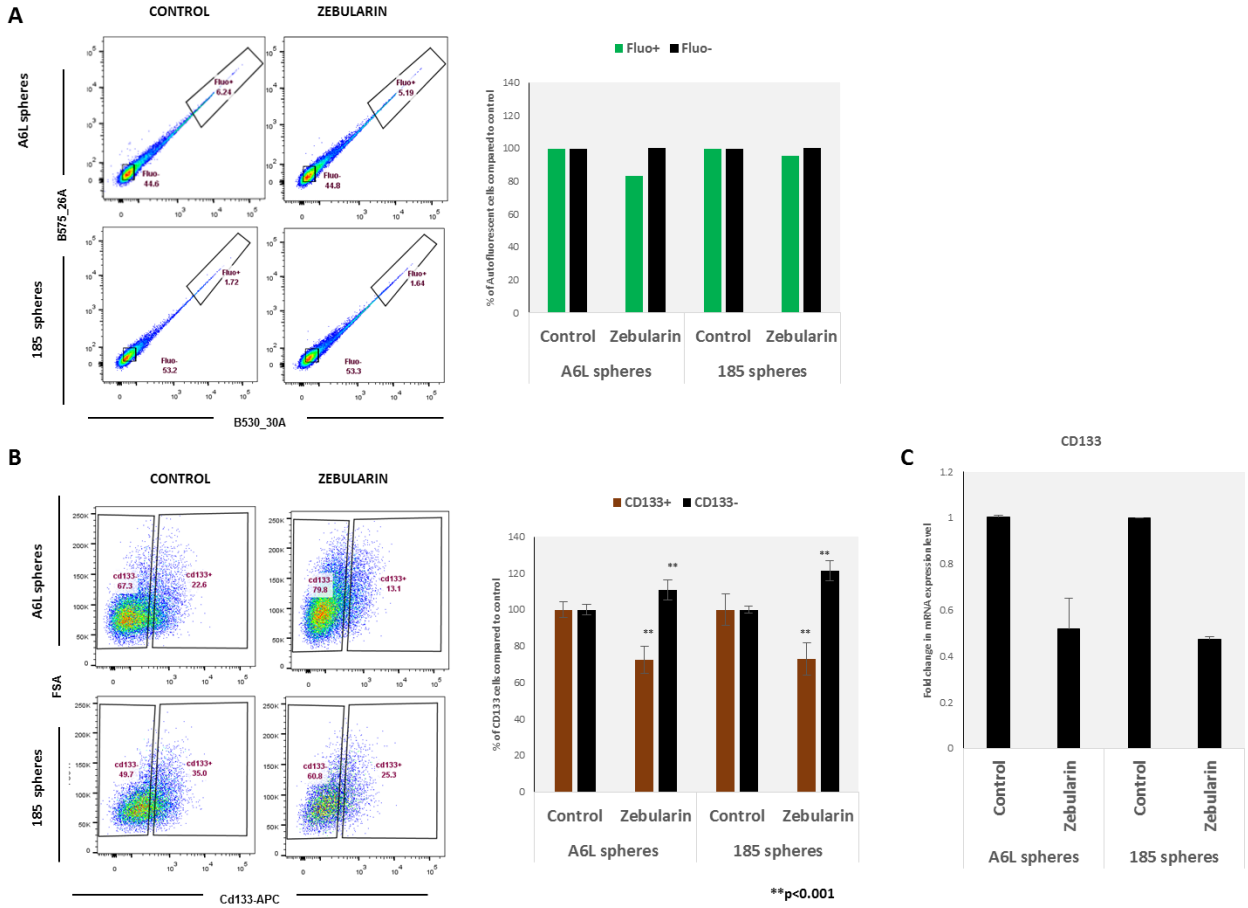

Figure 23. (**A**) Flow cytometry plots showing the percentage of autofluorescent-positive cells following 7 days of Zebularine treatment. Primary PDAC cells (A6L and 185) were plated in sphere culture conditions and treated with 75µM of Zebularine every 2nd day, after which we assessed the percentage of autofluorescence. The quantification of these results are presented in the right panel normalized to percentage of autofluorescence in untreated cells (**B**) Flow cytometry plots showing the percentage of CD133-positive and CD133-negative cells from PDAC spheres treated for 7 days with zebularine. The quantification of these results are presented in the right panel. Data are represented as percentage of CD133-positive/negative in untreated cells; graph show summary from three independent experiments. **(C)** RTqPCR analysis of CD133 mRNA in PDAC spheres-derived cells treated with zebularine for 7 days. Data are normalized to β-actin and represented as fold change compared to untreated cells.

The above detailed results suggested that zebularine is targeting the CSC population. Therefore, we next evaluated the effect of zebularine in specific CSC properties. In order to address if Zebularine treatment impairs CSC self-renewal, we assessed the ability of PDAC cells to form 1st generation and 2nd generation spheres in the absence or presence of 75µM zebularine. The sphere-derived cells treated with Zebularine displayed a significant decrease in self-renewal capacity, resulting in lower numbers spheres/ml in comparison to control-treated cultures (Figure 24A). Moreover, the expression of the pluripotency-associated genes *NANOG*, *OCT3/4*, *SOX2* and *KLF*4 were also decreased following treatment (Figure 24B).

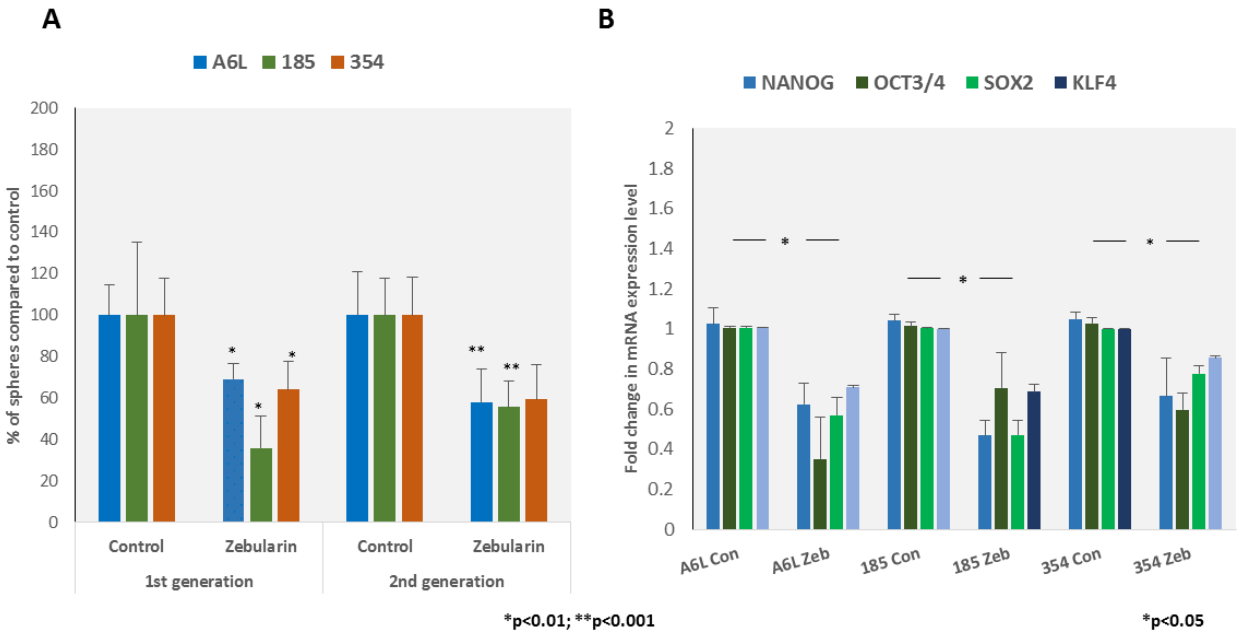

Figure 24. (**A**) Graph showing number of spheres in 1st and 2nd generation in various primary PDAC cells (A6L, 185 and 354) untreated and treated with 75µM of Zebularine. Low number of cells 2000 cells/ml were plated in triplicate for sphere formation, and treated with zebularine, every 2nd day. 1st generation spheres were counted after 7days or cells were dissociated and plated for 2nd generation for additional 7 days. Zebularine treatment was continued during 2nd generation sphere formation. Data are represented as percentage of counted spheres with size > 40µm compared to untreated cells. Results are summary of at least three independent experiments (**B**) RTqPCR analysis of pluripotency-associated genes (*NANOG*, *OCT3/4*, *SOX2* and *KLF4*) in 1st generation spheres treated for 7 days with zebularine. Data are normalized to β-actin and presented as fold change in compare to untreated cells.

3.4. Cancer stem cell survival and proliferation

In order to evaluate whether Zebularine's effect on CSCs was mediated via induction of cell death (i.e. apoptosis), we performed Annexin V staining on PDAC spheres treated with 75µM zebularine and observed no significant difference in Annexin V staining (i.e. early and late apoptosis) between control- and zebularine-treated spheres (Figure 25A). Moreover, since zebularine treatment was able to significantly decrease the percentage of CD133-positive cells (Figure 23B and 23C), we also looked at apoptosis induction specifically within the populations of CD133-positive and –negative cells and we observed that both populations of cells had the similar percentages of Annexin V-positive cells (Figure 25B). At the cell cycle level, DAPI analysis of A6L sphere-derived cells revealed an increase in G2/M residing cells; however, in 185 cells we observed no difference in the cell cycle distribution following zebularine treatment (Figure 26). Zebularine neither induced apoptosis nor altered the cell cycle state of treated cells, although the treatment could slightly increase percentage of G2/M residing cells in A6L spheres.

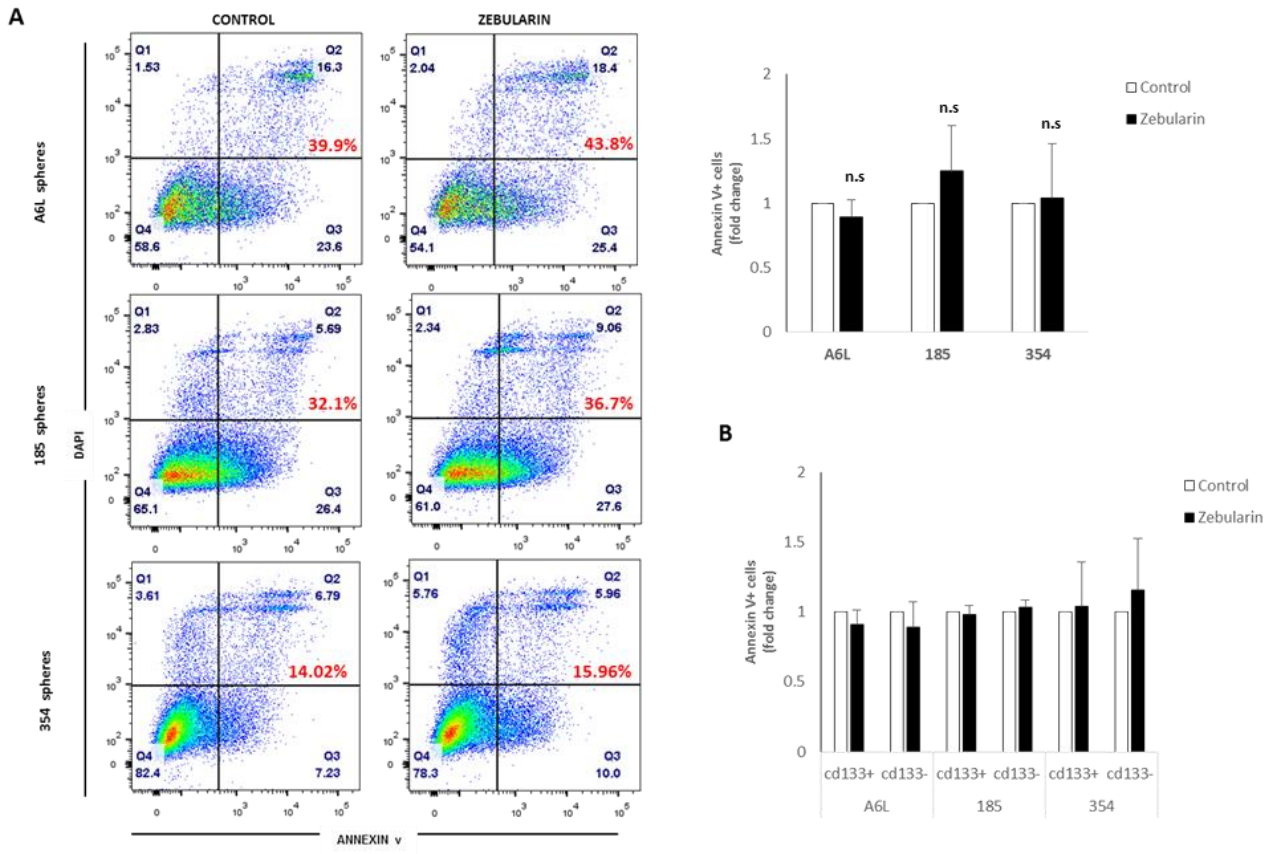

Figure 25. (**A**) Representative flow cytometry plots of Annexin V staining in various PDAC sphere-derived cells treated with zebularine. Cells were plated in sphere culture conditions and treated with zebularine every 2nd day. At day 3 cells were filtered through 50µm filters to take up only spheres with size higher than 40µm, trypsinized and stained with Annexin V antibody (left panel; red numbers on facs plots represent total percentage of Annexin V-positive cells). Right panel shows quantification of Annexin V positive cells from three independent experiments and data are represented as fold change in compare to untreated cells. (**B**) Graph showing quantification of Annexin V positive cells in CD133-positive and CD133-negative cells upon treatment with zebularine. Cells were treated and processed as described for spheres, except that staining for CD133 was done prior to Annexin V staining in order to assess cell death in marker +/- population. Data are represented as fold change in compare to untreated cells.

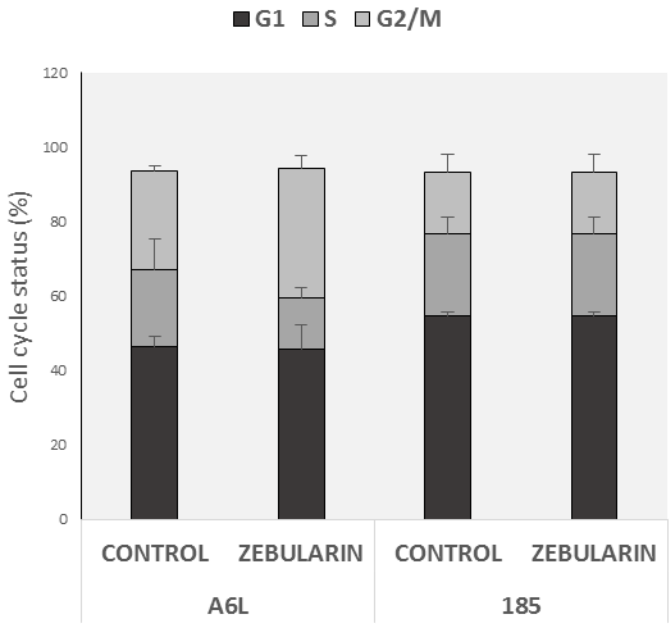

Figure 26. Graph showing cell cycle analysis using DAPI. Primary PDAC cells A6L and 185 were plated in sphere culture conditions and treated with 75μM of zebularine. At day 3 cells were filtered through 50μm filters and trypsinized. Cell cycle distributions was assessed by staining DNA with DAPI.

3.4. In vivo tumourigenicity

The most defining feature of CSCs is their ability to form tumours *in vivo*. After observing that zebularine treated cells had decreased sphere formation capacity and reduced expression of pluripotency-associated genes and stem cell markers, we next aimed to test their ability to form tumours in mice. After treating PDAC sphere-derived cells for 7 days with zebularine, we performed limiting dilution tumourigenicity assay by injecting serial dilutions of control or zebularine-treated cells into Nude mice and followed tumour formation over several months. Zebularine treated cells produced significantly fewer tumours as in a cell number-dependant manner compared to control cells (Figure 27). In line with our observations that zebularine targets CSCs, we observed that the frequency of CSCs was approximately 6- 5-fold lower in mice injected with zebularine-treated A6L and 354 PDAC cells, respectively.

Tumor take
(# tumors / # injections)

		10^3	10^2	Freq
A6Ls	Control	7/18	3/11	1/1507
	Zebularin	2/18	0/12	1/9091
354s	Control	12/12	5/12	1/503
	Zebularin	7/12	2/12	1/2420

Figure 27. Summary of in vivo tumourigenicity of subcutaneously-injected zebularine-treated cells and control sphere-derived cells 11-weeks post injection together with CSC frequency determined using the extreme limiting dilution analysis algorithm (http://bioinfo.wehi.edu.au/software/elda/index.html) right, 95% CI).

Moreover, after harvesting tumours we assessed the expression of markers such EPCAM, CD133 and CXCR4, and observed that tumours derived from zebularine-treated cells had a higher percentage of EPCAM-positive cells indicating a reduced desmoplastic reaction. More importantly, within the EPCAM-positive population we observed that tumours from zebularine-treated cells showed a marked decrease in the percentage of CD133-positive and CXCR4-positive cells, again indicating a reduction in the CSCs compartment following zebularine treatment (Figure 28).

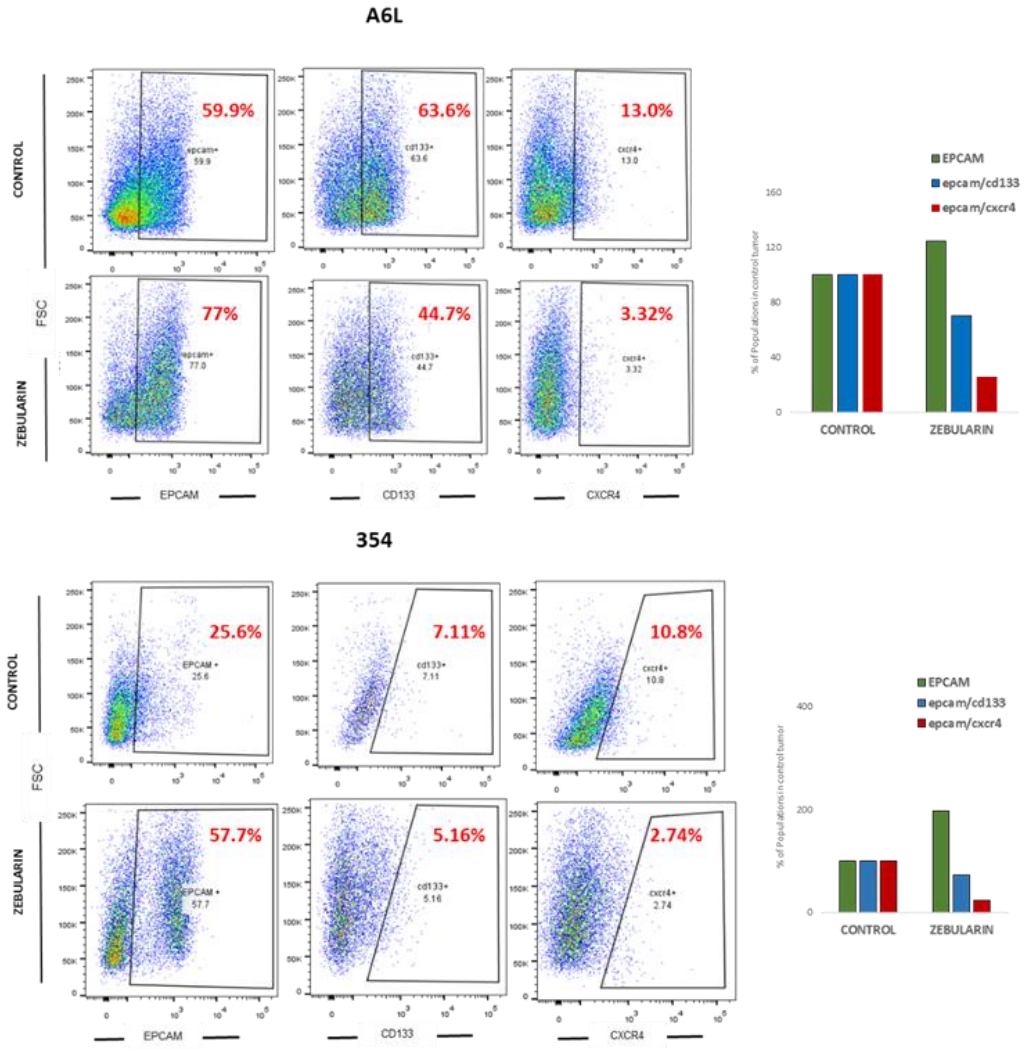

Figure 28. Representative flow cytometry charts showing percentage of EPCAM, CD133 and CXCR4 markers in tumours derived from zebularine treated cells and control cells. 11-weeks post injections tumours from zebularine and control treated sphere-derived PDAC cells A6L and 354 were explanted, digested and processed for flow cytometry analysis. Right panel shows quantification of EPCAM-positive tumour cells, and CD133-positive and CXCR4-positive cells inside EPCAM-positive population represented as percentage in compare to untreated cells.

4. DISSECTING THE MECHANISM BEHIND ZEBULARINE EFFECT ON PDAC CSCs

4.1. Zebularine demethylation signature

Given that zebularine decreased DNMT1 protein levels, and that zebularine-treated CSCs showed decrease "stem"-like features and impaired tumourigenic potential, we next set out to determine the potential mechanism(s) responsible for zebularine mediated effects on CSCs by first analysing global DNA methylation in zebularine-treated CSCs. We obtained the genome-wide methylation profile of zebularine-treated and -untreated sphere-derived cells using an Illumina Infinium HumanMethylation450 BeadChip. As shown in Figure 29, zebularine induced significant decrease in PDAC CSC global DNA methylation levels.

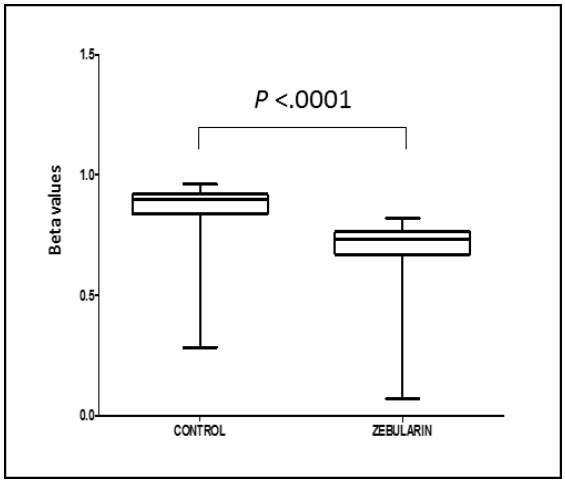

Figure 29. Box plot showing global decrease in DNA methylation in zebularine treated sphere cells compared to control. PDAC primary cells A6L and 185 were plated in sphere culture conditions and treated with zebularine every 2nd day for 7 days. Cells were trypsinized and subsequent DNA extracted from these cells have been used for methylation array. The box plot shows global methylation difference in pooled treated cells versus untreated cells.

One of the very interesting targets of DNA methylation are miRNA. miRNA are known to be involved in wide range of biological processes, such as cell cycle control, stem cell differentiation, hematopoiesis, hypoxia and ageing. Epigenetic factors, like DNA methylation can influence the expression of miRNA through hypermethylation of CpG islands that are located very close to several miRNA. Modulation of miRNAs via methylation can also be tumour specific and cell type

specific. For example, while miR-124a is hypermethylated and expressed at low levels in colorectal tumours, no methylation of miR-124a was seen in neuroblastoma where the expression of this miRNA were very high (Lujambio et al., 2007). A recent publication by Cioffi *et al*, showed miR-17-92 cluster to be down-regulated in chemoresistant CSCs versus non-CSCs (Cioffi et al., 2015). In gain of function experiments, the authors showed that forced overexpression of miR-17-92 reduced CSC self-renewal capacity, in vivo tumourigenicity and chemoresistance by targeting multiple NODAL/ACTIVIN/TGF-β1 signalling cascade members as well as directly inhibiting the downstream targets p21, p57 and TBX3. Specifically, the Nodal/Activin pathway is essential for the regulation of ''stemness'' properties of pancreatic CSCs. The ligands are expressed in pancreatic CSCs regulating self-renewal and metastasis (Lonardo et al., 2012). Building on these previous findings and considering that the reason for miR-17-92 downregulation in CSCs is still unknown, we wanted to investigate if DNA methylation plays a role in this regulation and if it can be modulated by Zebularine treatment.

4.2. Downregulation of miR-17-92 in CSCs could be due to hypermethylation

MiR-17-92 cluster is composed of six members: miR17,-18,-19a,-19b,-20b and -92a. As previously shown this cluster of miRNAs is down-regulated in PDAC CSC, but the mechanism(s) behind the differential regulation of this cluster in PDAC CSCs and non-CSCs is still unknown (Cioffi et al., 2015). Observations that CpG island could be found in close proximity of the miR-17-92 promoter encourage us to investigate whether hypermethylation of this CpG island could be responsible for downregulation of this important miRNA cluster in CSCs (Figure 30).

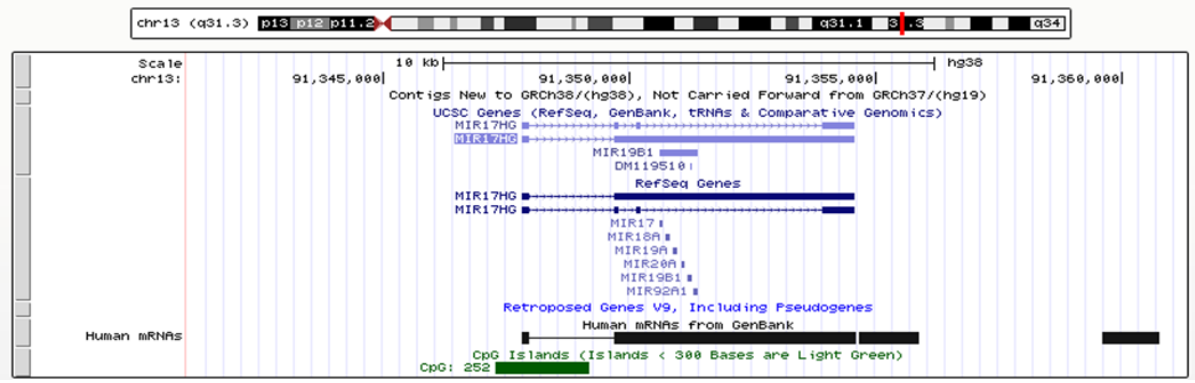

Figure 30. Screen shot of genomic locus coding for miR-17-92 miRNA cluster showing CpG island in proximity

We searched our methylation data obtained from zebularine treated spheres (Figure 31) and discovered that indeed all CG sites covered in our methylation array and in proximity of the miR-17-92 miRNA cluster had decreased methylation levels upon zebularine treatment. These data suggested that zebularine treatment could potentially unlock the epigenetic restriction of this cluster in CSCs, resulting in the expression of these repressed "anti-CSC" miRNAs.

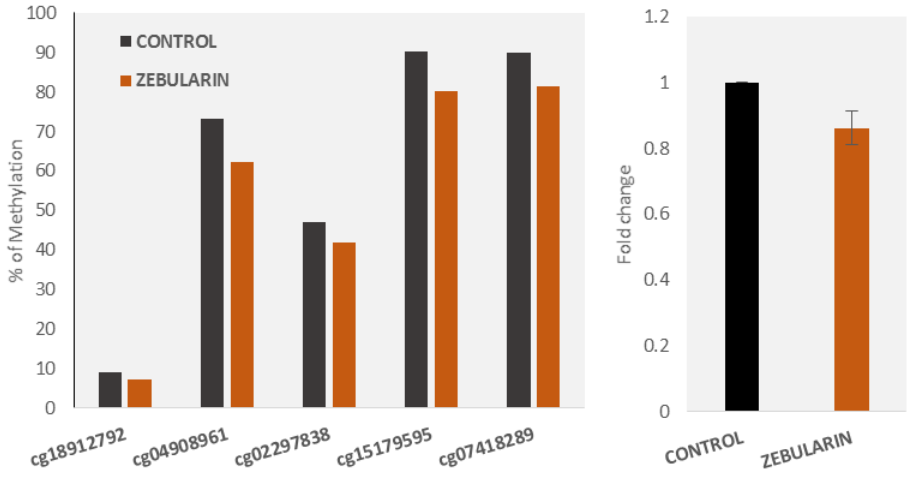

Figure 31. Graph showing methylation percentage of CpG probes adjacent to the miR-17-92 locus in zebularine treated cells compared to control cells (left panel) and average decrease of methylation across all CpG sites represented as fold change in compare to untreated cells (right panel).

To test the aforementioned hypothesis we assessed the expression of miR-17-92 members in an independent set of sphere-derived control- and zebularine-treated cells. We observed a notable increase in the expression of miR-17-92 members upon treatment and across a panel of PDAC sphere-derived cells. There was a particular and notable increase miR-19a and miR-19b compared to the other miRNA cluster members (Figure 32).

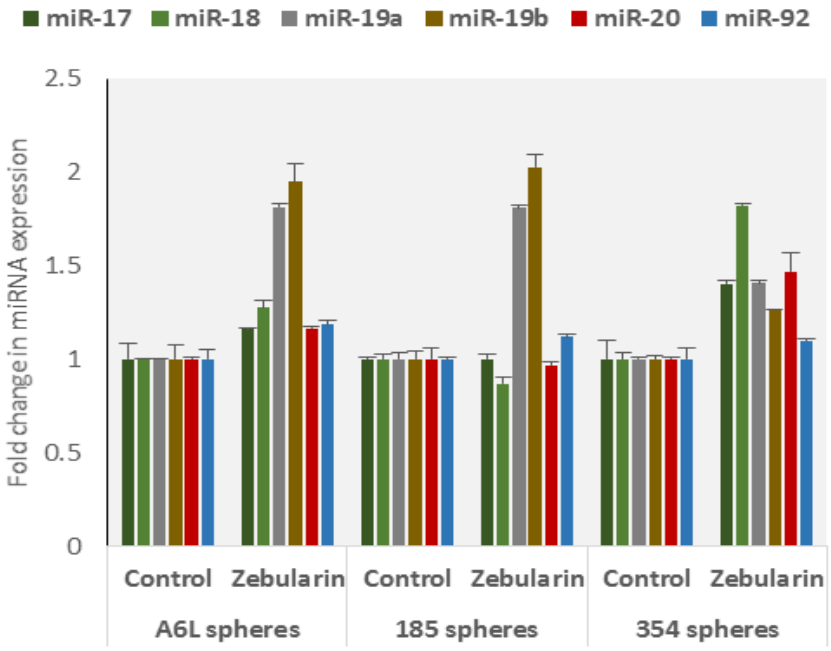

Figure 32. RTqPCR analysis of members of miR-17-92 cluster (miR17,-18,-19a,-19b,-20,-92) in primary PDAC spheres A6L, 185 and 354 treated with zebularine for 7 days. Data are represented as fold change in compare to untreated cells.

4.3. Zebularine treatment influence the expression of miR-17-92 target genes

Several members of the miR-17-92 cluster have been shown to target diverse pathways, including TGF-β1 and HIF-1α signalling. Cioffi *et al*, identified several targets clustering around pathways that have been previously associated with CSCs regulation and ‘’stemness’’ (e.g. NODAL/ACTIVIN pathway). Moreover they showed that some members of this cluster can also target genes such as p21 and Tbx3 (Cioffi et al., 2015)

We observed that zebularine treatment could decrease the expression of miR-17-92 target genes such as p21, TGFBR2, ALK4, Smad2 and Smad4 (Figure 33A), similarly to lentiviral-mediated overexpression of this cluster shown by Cioffi *et al.* (Cioffi et al., 2015). Moreover zebularine treatment decreased p21 protein levels, confirming the results observed at the mRNA level (Figure 33B).

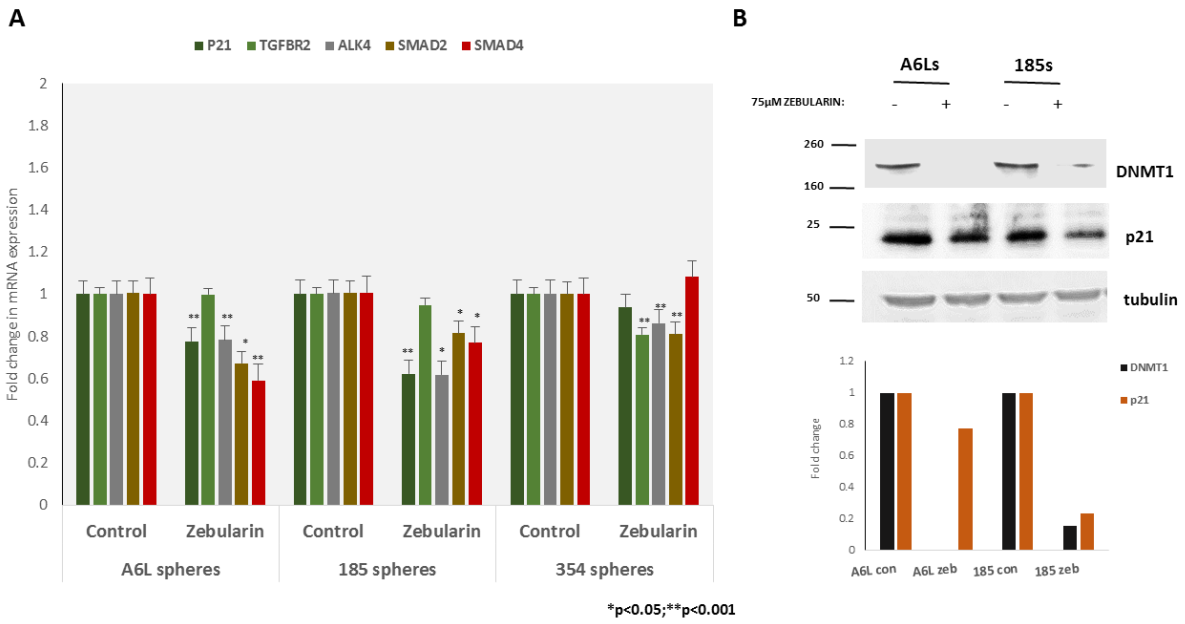

Figure 33. (A) RTqPCR analysis of miR-17-92 target genes (p21, TGFBR2, ALK4, SMAD2 and SMAD4) in various PDAC sphere-derived cells (A6L, 185 and 354). Cells were plated in sphere culture conditions and treated with zebularine every 2nd day for 7 days. Data are normalized to β-actin and represented as fold change in compare to untreated cells. **(B)** Western blot showing the DNMT1 and p21 protein levels in PDAC sphere-derived cells A6L and 185 treated with zebularin for 7 days. Protein levels were normalized to tubulin and subsequently quantified by densitometry. Change in protein level are represented as fold change in compare to untreated cells.

In order to provide more direct proof that some of the effects observed with zebularine could be mediated via hypomethylation of the miR17-92 cluster, resulting in the expression of this normally silent miRNA cluster in CSCs, we decided to perform loss of function experiment in the presence of zebularine. As previously shown, members of the miR-17-92 cluster are down-regulated in CSCs, and overexpression of this miRNA cluster impairs CSC properties. On the other hand, downregulation of this cluster (i.e. inhibition of miR17-92 using antagomirs) in more differentiated cells shows the opposite effect, imparting on non-CSCs cancer stem cell-like phenotypes, such as increased self-renewal (i.e. increased sphere formation). We therefore treated differentiated cells for 24h with antagomiRs targeting different members of cluster. Following treatment we plated cells in conditions that promote cancer stem cell expansion (e.g. sphere formation assay) and subsequently started treatment with zebularine to competitively reverse the effects of miRNA downregulation. As it can be seen in Figure 34, zebularine treatment could abrogate the sphere formation enhancement induced by antagomir treatment, and moreover this effect was most obvious when zebularine was added to cells previously treated with antagomiRs for miR-17,miR-

18, -19a/b and -20. Thus, we can conclude that zebularine mediates its inhibitory effects on CSCs, in part, by inducing the expression of the members of the miR-17-92 cluster.

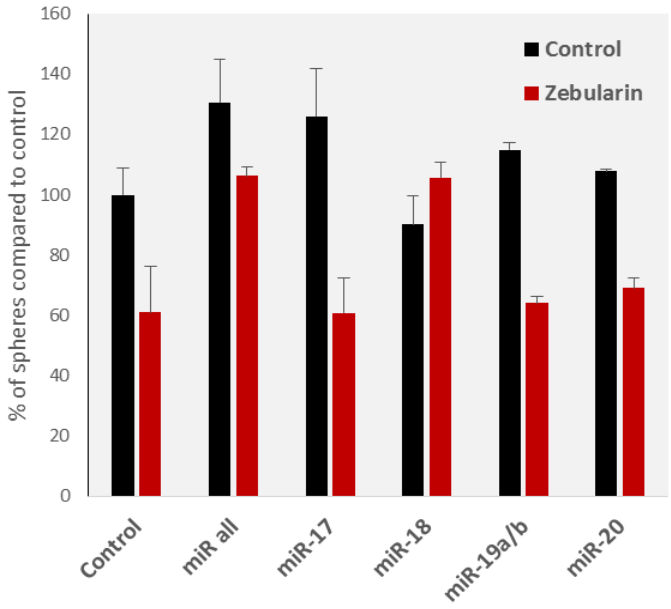

Figure 34. PDAC 185 cells were plated in adherent conditions and 24h latter treated with antagomiRs for miR-17, -18, -19a/b and -20 or all miRNAs. Twenty four hours after cells were trypsinized and low number of cells 2000 cells/ml were plated for sphere formation in triplicate and treated with zebularine every 2nd day for 7 days. Data are represented as percentages of spheres with size > 40µm in compare to untreated control.

5. ROLE OF DNA DEMETHYLASES IN PDAC CSC

In order to characterize specific genes/pathways that influence tumourigenicity and metastatic potential of pancreatic CSC, RNA-seq was performed on multiple PDAC primary cells cultures grown under sphere and adherent conditions. This project, governed by other members from our group, identified common signatures of up-regulated and down-regulated genes. Focussing on the up-regulated group of genes, our attention caught one group of genes recently identified as very important players in shaping the epigenetic landscape of cells. These genes belong to the family of TET proteins. TET proteins are Fe (II) and 2-oxoglutarate (2OG)-dependent dioxygenases that successively oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), thereby mediating active DNA demethylation (He et al., 2011; Pastor et al., 2013; Tahiliani et al., 2009). Since the sum of our aforementioned studies strongly support an important role for DNA methylation in CSC biology, including a specific role for DNMT1 in regulating the "stemness" of CSCs in PDAC, we next set out to determine if TET proteins also play a critical role in PDAC CSC "stemness" and biology.

5.1. TET proteins are overexpressed in spheres versus adherent cells

There are three different TET genes, TET1, TET2 and TET3 (Pastor et al., 2013). TET1 and TET2 have been extensively described as having functional roles in ESC and only recently they have been shown to be involved in cancer (Hsu et al., 2012; Kim et al., 2011; Sun et al., 2013b). TET3, however, has been primarily studied in the context of demethylation in the paternal genome (Gu et al., 2011). We therefore focussed our attention on TET1 and TET2 in PDAC CSCs. There are two isoforms of TET2 in humans: TET2.1 and TET2.2. The human TET2 isoform 2 is a 130 kD truncated protein missing the C-terminal 836 amino acids compared to the full-length protein that contains a unique C-terminal sequence (Scourzic et al., 2015). To date, however, the function of the TET2 isoform is not fully understood. We initiated our studies by first checking the expression of TET1 and the two TET2 isoforms in independent sets of sphere-derived and adherent PDAC cultures. In concordance with our RNA-seq data, we observed significant up-regulation of TET1 and both TET2 isoforms in spheres versus adherent cultures (Figure 35A). Moreover CSCs isolated using markers such as autofluorescence and CD133 (autofluorescent-positive and CD133-positive) showed

significant up regulation of TET genes compared to non-CSCs (autofluorescent-negative and CD133-negative) (Figure 35B).

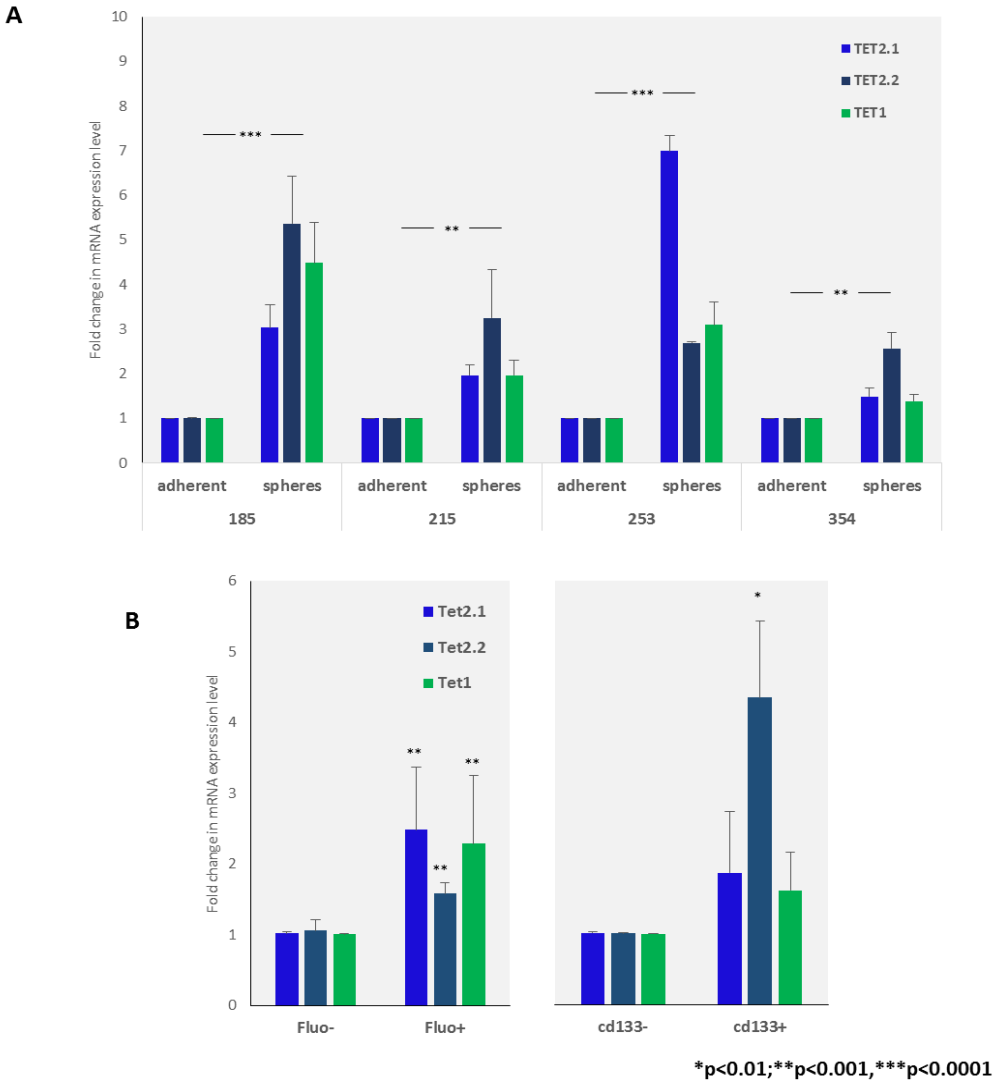

Figure 35. (A) RTqPCR analysis of TET genes (Tet1, and two isoforms of Tet2 gene -Tet2.1 and Tet2.2) in adherent cells versus spheres from various primary PDAC cells (185, 215,253 and 354)**. (B)** RTqPCR analysis of TET genes (Tet1, Tet2.1 and Tet2.2) in autofluorescent-positive (Fluo+) and -negative (Fluo-) cells from PDAC 185 adherent cells or CD133-positive and CD133-negative cells sorted from PDAC 354 adherent cells. Data are normalized to β-actin and are presented as fold change in gene expression relative to adherent cells or CSC negative counterparts (Fluo- and CD133-).

We next evaluated the TET2 expression at the protein level by western blot analysis and to our surprise we saw that TET2 protein levels were lower in sphere-derived cultures versus adherent cultures (Figure 36A). A similar trend was observed when we compared FACS sorted autofluorescent-positive and -negative cells or CD133-positive and -negative cells (Figure 36B).

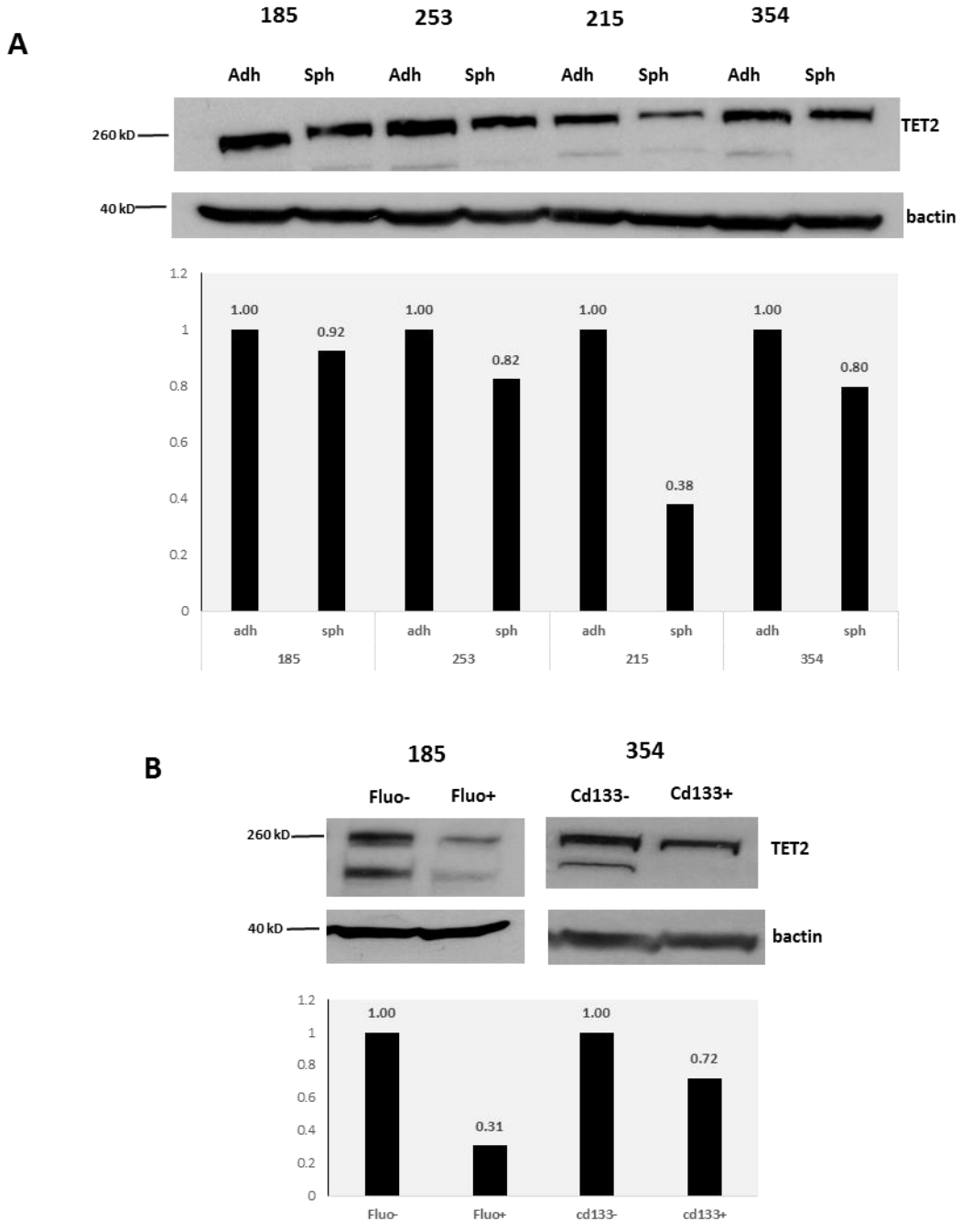

Figure 36. **(A)** Western blot analysis for TET2 protein level in 7days old spheres versus adherent cultures from panel of PDAC primary cells (185,253,215 and 354). Protein levels were normalized to β-actin and subsequently signal quantification was performed by densitometry; changes in protein level are represented as fold change compared to adherent cells**. (B)** Western blot analysis for TET2 protein level autofluorescent-positive vs. autofluorescent-negative cells sorted from PDAC 185 adherent cells or CD133-positive vs. CD133-negative cells FACS sorted from PDAC 354 adherent cells. Protein levels were normalized to β-actin and quantification was performs by densitometry. Change in protein level are represented as a fold change compare to negative counterparts (Fluo- and CD133-)

According to the literature, TET2 protein expression is markedly reduced in a wide range of solid cancers, including melanoma, prostate, breast, pancreas and liver tumours when compared with the matched surrounding normal tissues (Yang et al., 2013a). Moreover, TET2 is frequently inactivated by mutation in myelodysplastic syndromes from which leukemias can arise (Delhommeau et al., 2009; Langemeijer et al., 2009) and TET2 loss in mice was shown to results in increased hematopoietic stem cell self-renewal and transformation (Moran-Crusio et al., 2011).

5.2. TET2 inhibition using lentiviral knockdown approach promotes CSC phenotypes

To our surprise we found no correlation between TET2 mRNA expression and TET2 protein levels when we compared PDAC CSCs to non-CSCs (adherent versus spheres or CSC marker positive/negative cells). While perplexing at first, most publications as mentioned, investigating the role of TET2 in cancer report decreases in TET2 protein levels during cancer progression (Yang et al., 2013a). Crusio *et al.,* showed that in mice the loss of TET2 in the hematopoietic compartment led to progressive myeloproliferation *in vivo*, with features characteristic of human CMML (Moran-Crusio et al., 2011). In addition, TET2 haploinsufficiency confers increased self-renewal to stem/progenitor cells and to extramedullary hematopoiesis. These studies therefore prompted us to perform loss of function experiments by using GFP reporter lentiviral constructs with shRNAs against TET2 in more differentiated (adherent) cells where TET2 protein expression is high (Figure 36).

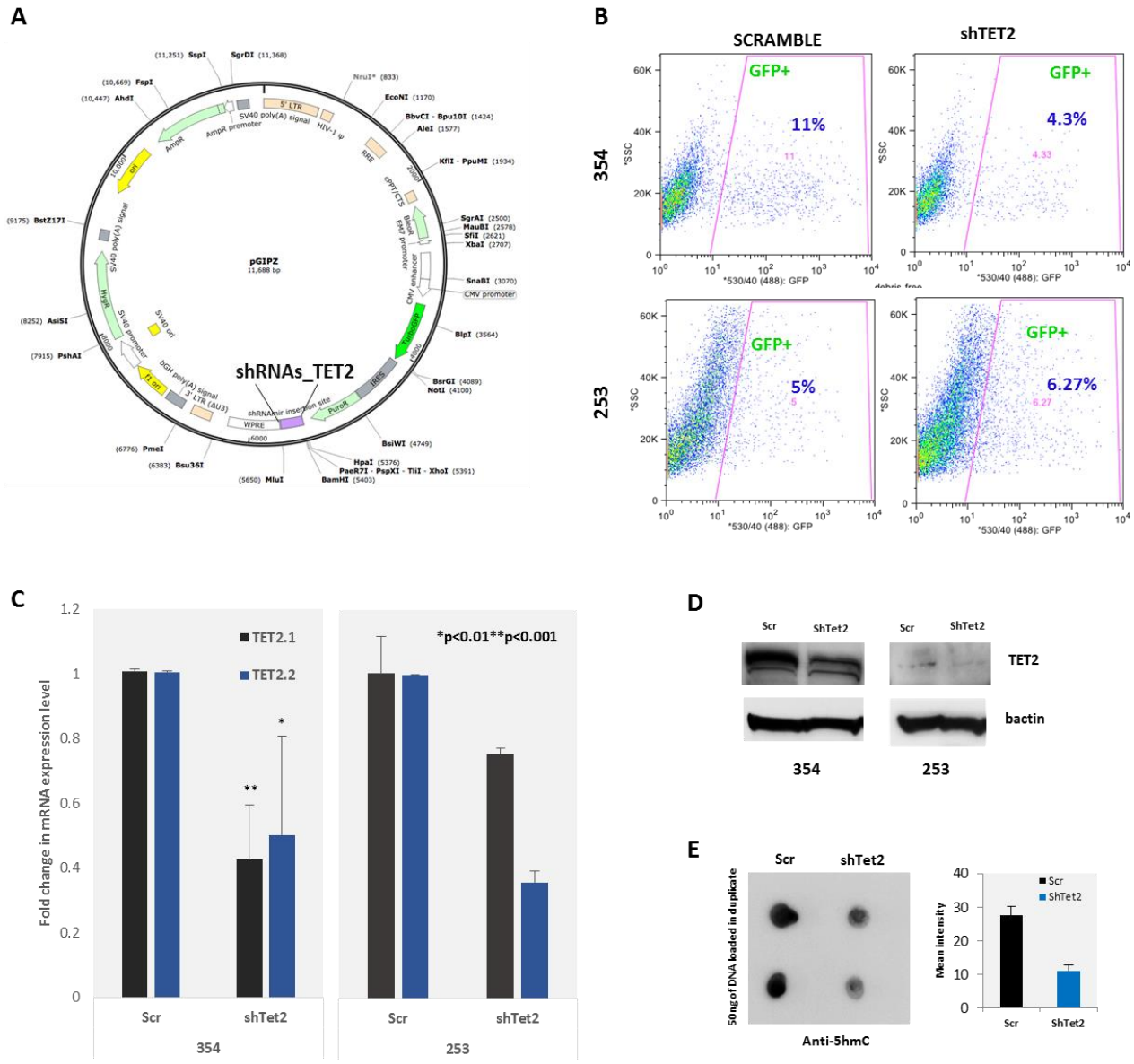

Figure 37. **(A)** Map of pGIPZ lentiviral vector containing shRNAs targeting TET2 gene and turboGFP marker for selecting cells that contain construct with shRNATet2. **(B)** Flow cytometry plots showing sorting strategy based on GFP, in adherent PDAC 354 and 253 cells, used for high purification of lentiviruse-containing scramble and shRNA Tet2 cells 72h after transfection **(C)** RTqPCR analysis of TET2 gene in PDAC 354 and 253 cells sorted for lentiviral vector contacting scramble and shTet2 lentiviral construct with GFP. Data were normalized to β-actin and represented as fold change in compare to scramble cells **(E)** Western blot analysis of TET2 protein in PDAC 354 and 253 adherent cells sorted for GFP **(E)** Dot blot analysis of TET2 catalytic product 5hmC in PDAC 354 scramble and shtet2 adherent cells sorted for GFP; 50ng of DNA was manually applied onto Hybond N+ nylon membrane in duplicate and incubated with antibody for 5hmC. After visualization by chemiluminescence subsequently signal quantification was performed by densitometry.

Following infection of two primary PDAC adherent cultures with our shTET2 lentiviral vector at an MOI of 2.5 i.u./cell, cells were sorted for GFP and following expansion *in vitro*, we confirmed down-regulation of TET2 at the mRNA and protein level. In both primary PDAC adherent cultures, we observed down-regulation of TET2. Moreover, we checked the level of the TET2 product 5hmC and observed that in TET2-silenced cells there was a decrease in global 5hmC levels. Several previous studies also showed reduction of 5hmC levels upon down-regulation of TET2 in different cells types (Ko et al., 2010; Pronier et al., 2011). Having established cultures with constitutive and functional silencing of TET2, we next performed several experiments to elucidate the effect of TET2 silencing at the level of CSC phenotypes.

5.3. Cancer stem cell phenotypes

To see if down-regulation of TET2 influences CSC characteristics we first check the expression of pluripotency-associated genes (*NANOG, OCT3/4, SOX2* and *KLF4*) by RTqPCR. While, we saw no difference in the expression of *NANOG, OCT3/4* or *KLF4*, we did observe a significant reduction of *SOX2* mRNA expression in both primary PDAC adherent ShTet2 cells (Figure 38A). TET2 together with TET1 were shown to be highly expressed in murine and human ESC (Ito et al., 2010; Koh et al., 2011; Ruzov et al., 2011) indicating their role in regulating pluripotency. However, as observed in our study except for *SOX2* gene, down-regulation of TET2 in various ESC lines did not alter the expression of pluripotency markers (Koh et al., 2011). Using flow cytometry we next assessed the expression of the CSC marker CD133 and observed a consistent but not significant increase in the percentage of cells expressing CD133 in ShTet2 cells compared to control cells (Figure 38B).

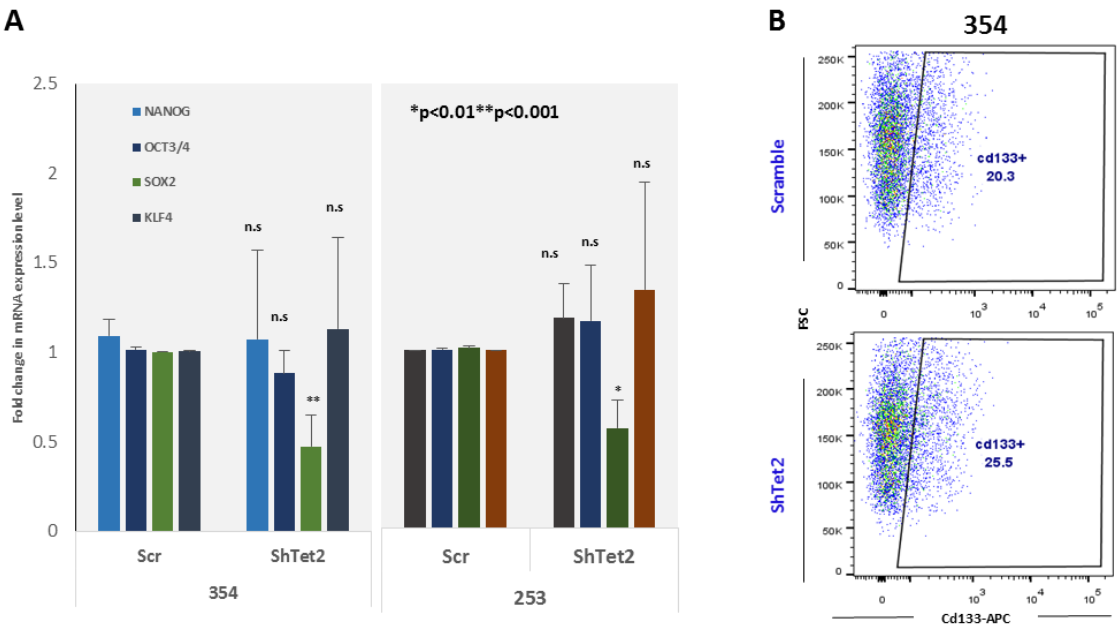

Figure 38. **(A)** RTqPCR analysis of pluripotency-associated genes (Nanog, Oct3/4, Sox2 and Klf4) in PDAC 354 and 253 adherent cells containing scramble and shTet2 construct after sorting for GFP. Data were normalized to β-actin and represented as fold change in compare to scramble cells. **(B)** Flow cytometry plot showing expression of marker CD133 in PDAC 354 scramble and shTET2 adherent cells sorted for GFP.

While the effects at the level of gene expression and CD133 expression were subtle and not entirely conclusive, we observed that down-regulation of TET2 in adherent cells had a more profound enhanced effect at the level of self-renewal capacity as determined by increased sphere formation (Figure 39A) and increased colony formation (Figure 39B). Increase in ‘’stemness’’ upon Tet2-scilencing was shown in hematopoietic system, where TET2 loss leads to increased stem cell self-renewal *in vivo* as assessed by competitive transplant assays (Moran-Crusio et al., 2011).

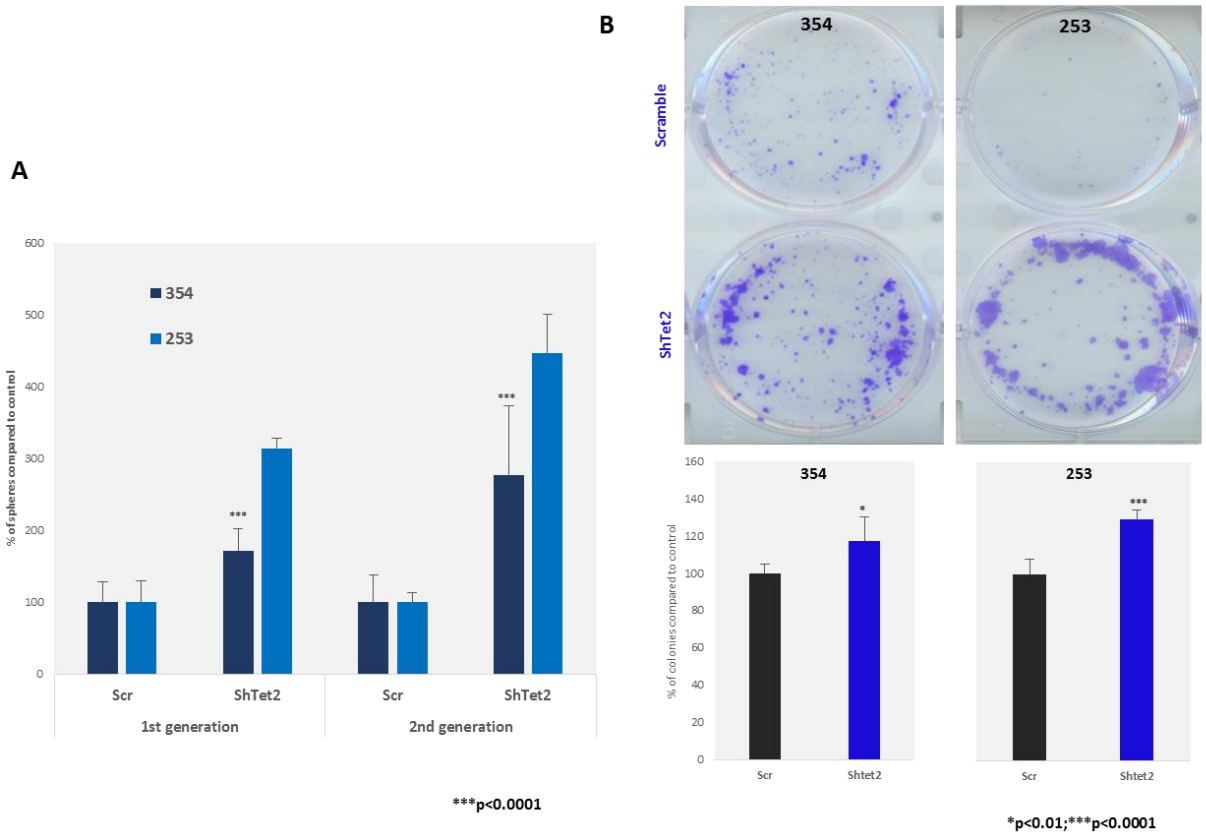

Figure 39. **(A)** Graph showing number of spheres in 1st and 2nd generation in scramble and shTet2 cells. After sorting adherent PDAC 354 and 253 cells for GFP, low number of cells 2000 cells/ml were plated for sphere formation (1st generation spheres were counted after 7days or cells were dissociated and plated for 2nd generation for additional 7 days). Data are represented as percentage of counted spheres with size > 40µm in shTet2 cells compared to scramble cells **(B)** Colony formation in 354 and 253 PDAC scramble and shTet2 adherent cells (upper panel) together with quantification in two independent experiments done in triplicate (down panel). Quantification was done by measuring absorbance of crystal violet upon lysis of colonies with 0.1% of SDS; data are represented as percentage of absorbance in shTet2 cells compared to scramble cells.

This marked increase in sphere formation and colony formation was not due to increased overall survival of ShTet2 cells as we found no difference in the percent of Annexin V positive cells in both adherent and sphere-derived cells (Figure 40). Thus, the effects observed were indeed due to increased self-renewal capacity *in vitro*, suggesting that TET2 silencing affects this CSC property.

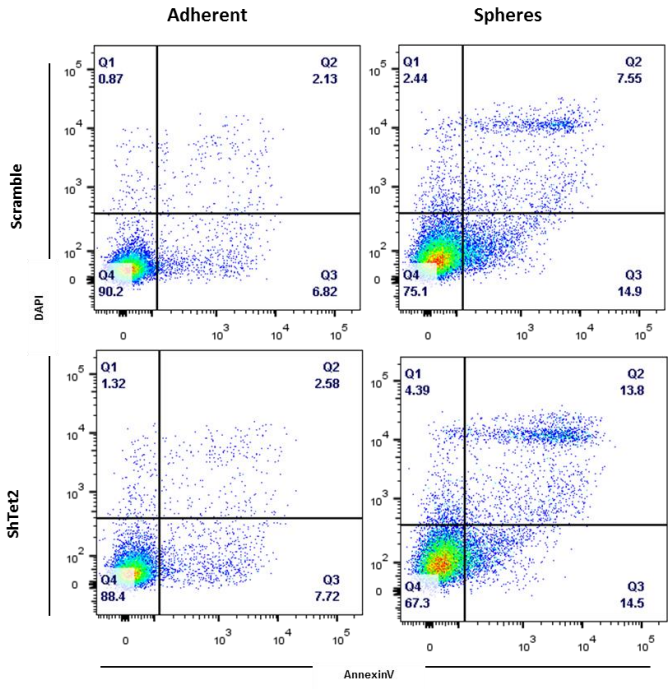

Figure 40. FACS plots showing survival of 354 PDAC scramble and shTet2 cells sorted for GFP. After sorting and *in vitro* expansion cells were seeded in adherent and sphere culture conditions and percentage of Annexin V/DAPI positive cells were measured at day 3. Experiment was done in duplicate (n=2)

5.4. In vitro migration capacity

Many previously published studies highlighted the existence of a small subpopulation of tumourigenic cancer cells, (CSCs), with metastatic potential (Malanchi et al., 2012). Some reports demonstrate that CSCs are enriched among circulating tumour cells in the peripheral blood of patients with breast cancer (Armstrong et al., 2011) and these studies show that epithelial-mesenchymal transition (EMT), an early step of tumour cell migration, can induce differentiated cancer cells into a CSC-like state (van der Horst et al., 2012). TET proteins were recently reported to play a role in cancer cell invasion and migration. Hsu *et al*, showed that enforced expression of TET1 reduced cell invasion and breast cancer xenograft tumour formation (Hsu et al., 2012). Moreover Song *et al*, have shown that TET2 can promote EMT and ‘’stemness’’ in breast cancer through regulation of miRNAs (Song et al., 2013b). These studies promoted us to check the migrating capacity of primary pancreatic cancer cells after down-regulation of TET2. Using wound healing assay to measure the migrating capacity of transduced cells, we observed that ShTet2 cells

were able to close the wound faster than control cells (Figure 41), suggesting that these cells could have more aggressive invasive characteristics.

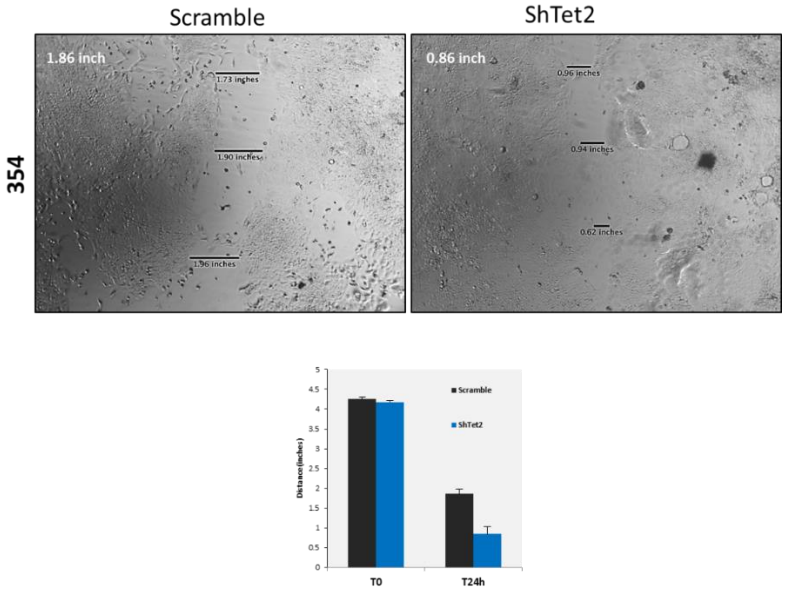

Figure 41. Representative images of scratch wound healing assay of 354 PDAC scramble and shTet2 adherent cells. Adherent cells were sorted for GFP to select for cells containing lentiviral scramble and shTet2 construct. After *in vitro* expansion cells were seeded in 6 multi-well plates in triplicate. Confluent cultures were scratched and migration was evaluated 24h later by calculating the average size of the wound determined by measuring the size of the wound at three locations (n = 3 wounds per cell/condition).

5.5. In vivo tumourigenicity

Based on our *in vitro* results demonstrating that TET2 silencing in more differentiated cells confers upon these cells "CSC-like" characteristics, such as increased sphere formation ability, increased colony formation and increased expression of cell surface CSC markers (i.e. CD133), we proceeded to test the tumourigenic capacity of TET-silenced PDAC cells. 354 PDAC adherent scramble and shTet2 cells were sorted for GFP and injected subcutaneously in limiting dilution manner into the flanks of nude mice. Tumour onset and growth was followed for 11 weeks, after we observed that shTet2 cells had higher tumourigenicity and CSC frequency than scramble cells (Figure 42A). Moreover, during tumour follow-up we observed that shTet2 cells had higher take rate which also translated into formation of larger tumours in compare to scramble cells (Figure 42B). After digestion of tumours derived from shTet2 and scramble cells, we performed flow cytometry analysis and interestingly we observed increase in CSC markers CD133 and CXCR4 in

tumours derived from shTet2 in compare to tumours derived from scrambled cells (Figure 42C).

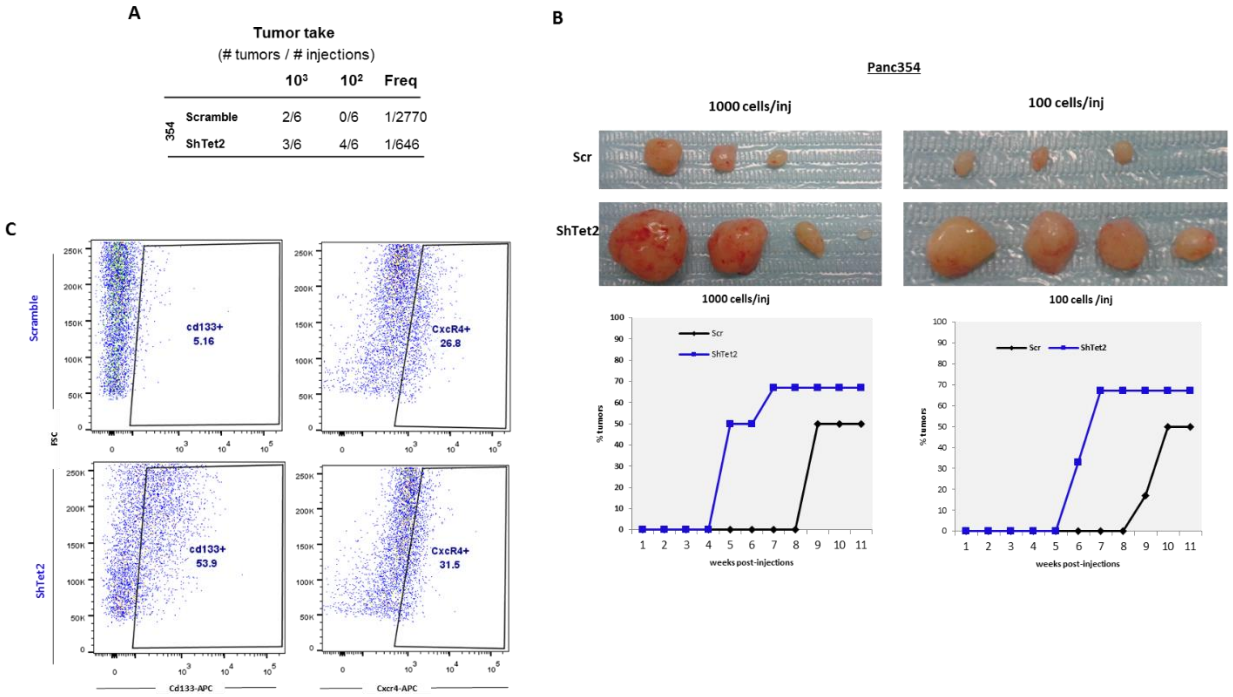

Figure 42. (A) Summary of in vivo tumourigenicity of subcutaneously-injected 354 PDAC adherent shTet2 and scramble cells after 11-weeks post injection together with CSC frequency determined using the extreme limiting dilution analysis algorithm (http://bioinfo.wehi.edu.au/software/elda/index.html) **(B)** Images of resected tumours derived from shTet2 and scramble cells (upper panel) after 11-weeks; graphs showing tumour take rate during 11-week follow up of tumour growth (down panel). Data are represented as percentage of observed tumours with volume higher than $50mm^3$ in every week compared to total expected tumours **(C)** FASC plots showing percentage of CSC markers, CD133 and CXCR4 in GFP positive tumour cells derived from shTet2 and scramble cells.

DISCUSSION

Of all human malignancies, pancreatic cancer is considered to be one of the most aggressive. Unfortunately, pancreatic cancer is contended to be a systemic disease at presentation, and as such it is unfortunately incurable, has a minimal chemotherapeutic response profile, and a high propensity for local, regional and distant metastasis (Garrido-Laguna and Hidalgo, 2015). These properties may be in part attributable to the existence of CSCs within the tumour (Rasheed and Matsui, 2012). Indeed, stem cells have been implicated in pancreatic cancer progression, prognosis, and resistance to therapy, and as such, they represent crucial therapeutic targets.

Previous studies from our and other laboratories suggest that pancreatic cancer harbour a distinct subpopulation of putative CSCs defined by their self-renewal capacity; exclusive in vivo tumourigenicity (Hermann et al., 2007; Li et al., 2007), and ability to drive metastasis (Hermann et al., 2008). Hermann *et al*, showed that primary pancreatic CSCs can be enriched for *in vitro* as anchorage-independent spherical colonies termed spheres or via enrichment using the cell surface marker CD133 (Hermann et al., 2007). Furthermore, recent work by Miranda-Lorenzo *et al*, also identified a distinct autofluorescent sub-population of self-renewing and highly tumourigenic CSCs in numerous epithelial tumours, including PDAC (Miranda-Lorenzo et al., 2014).

This doctoral thesis aimed to study the epigenetic landscape of pancreatic CSCs. Thus, prior to initiating such studies, we first had to define the most effective and robust method for identifying and isolating CSCs from patient-derived PDAC xenograft cultures. As a first approach, we assessed the efficiency of CSC enrichment from anchorage-independent cultures (e.g. spheres). We analysed sphere-derived cells from three primary PDAC cell cultures (i.e. A6L, 186 and 354) and confirmed that sphere-derived cell expressed significantly higher mRNA levels of pluripotency-associated genes (e.g. *NANOG*, *SOX2* and *KLF4*) and higher protein levels of the pluripotency-associated regulator Nanog (Figure 1A and 1B). However, we did observe that *OCT3/4* was not among the pluripotency-associated genes significantly up-regulated across the tumours analysed in CSC-enriched sphere culture conditions. In addition, PDAC354 sphere-derived cells showed inconsistent gene expression patterns. Even though sphere culture conditions do enrich for CSCs and this methodology has been efficiently used by others (Hermann et al., 2007; Lonardo et al., 2011), anchorage-independent conditions can also promote the proliferation and subsequent differentiation of CSCs. Thus apart from CSCs there also exist CSC-derived progenies in different stages of the differentiation process. Consequently, it is not surprising that

the degree of CSC enrichment, as determined by pluripotency gene expression, can vary depending on the final cellular makeup of the sphere cultures analysed.

Moreover, as a consequence of enriching for CSCs, sphere-derived cultures also over-express CSC markers, such as CD133 (Figure 2A). In fact, we show that CD133-positive cells isolated from sphere cultures by FACS express higher mRNA and protein levels of Nanog compared to their CD133-negative counterparts (Figure 2B). While an effective approach for enriching for and subsequently isolating CSCs, the limitation of cell surface markers is that their expression can frequently be altered in response to different culture environments, such as *in vivo* xenografting or *in vitro* cell culture (Miranda-Lorenzo et al., 2014). Fortunately, Miranda-Lorenzo *et al*, recently identified a cell-surface marker-independent approach for isolating CSCs from various epithelial tumours using an inherent autofluorescent phenotype expressed exclusively in CSCs (Miranda-Lorenzo et al., 2014). Specifically, the authors demonstrated that autofluorescent-positive cells isolated across different human tumour entities exhibited a range of CSC phenotypes and properties compared to autofluorescent-negative cells. We were able to confirm these data, and reproducibly show that autofluorescent positive cells significantly overexpressed the pluripotency-associated genes *NANOG, OCT3/4, SOX2* or *KLF4* at both the mRNA and protein levels (Figure 4A and 4B).

Since autofluorescence allowed for the reproducible isolation of "stem-like" CSCs independent of the use of cell-surface markers, we chose to use this marker to separate cancer stem cells from non-cancer stem cells throughout our studies.

1. CHARACTERIZATION OF DNA METHYLATION LANDSCAPE IN PDAC CSCs

Recent advances in stem cell biology have shed light on how cells may evolve to acquire malignant characteristics during tumourigenesis (Wongtrakoongate, 2015). This oncogenic evolution of cancer stem and progenitor cells, which often associates with aggressive phenotypes of the tumourigenic cells, is controlled in part by dysregulated epigenetic mechanisms including aberrant DNA methylation leading to abnormal epigenetic memory.

Epigenetic modifications of DNA and histones, the core components of chromatin, constitute an additional layer of information that influences the expression of specific genes. DNA methylation, the addition of a methyl group to a cytosine base, is an epigenetic modification that

is evolutionarily ancient and associated with gene silencing in eukaryotes. Because, CSCs and non-CSCs probably share a very similar genetic background, epigenetic changes are likely largely responsible for the differences between these highly tumourigenic cells and their non-tumourigenic progeny. DNA methylation has been proposed as a key regulator of embryonic stem cells (ESCs) pluripotency or adult stem cells self-renewal and differentiation programs (Bird, 2002). It has been widely reported that maintenance of the pluripotency state is conferred by a set of development associated transcription factors -such as *OCT4, NANOG,* and *SOX2*- that occupy promoters of active genes associated with self-renewal. Expression of the aforementioned transcription regulators is usually controlled by CpG promoter methylation, and differentiation is accomplished by partial or full methylation of pluripotency-associated genes, resulting in their down-regulation (Berdasco and Esteller, 2011). Similar mechanisms may account for the observed expression of pluripotency-associated genes in cancer stem cells, while these genes are strongly down-regulated, or even completely silenced (*NANOG,OCT4*) in their differentiated progenies. Since autofluorescent-positive CSCs expressed higher levels of the pluripotency genes compared to autofluorescent-negative cells (Figure 4A and 4B), we asked whether DNA methylation could be responsible for the differential expression of pluripotency-associated genes, such as *NANOG*. We therefore performed bisulfite sequencing of two regions of the *NANOG* promoter that were chosen based on previous findings (Freberg et al., 2007; Kim et al., 2013), where it was shown that the methylation status of CpG dinucleotides covered by these regions changes during differentiation or reprograming. We observed that the average DNA methylation levels of both investigated regions were very similar between autofluorescent-positive and -negative cells (Figure 6A), however, when we looked at individual CpG sites, we observed that in Fragment 1, 6 out of 9 CpG dinucleotides had lower methylation levels in autofluorescent-positive than in autofluorescent-negative cells. Differences in Fragment 2 were less obvious (Figure 6B). These results could be interpreted in two opposing ways. 1) Because the average methylation of both investigated regions was similar between autofluorescent-positive and -negative cells, DNA methylation may not be the primary mechanism controlling the differential expression of *NANOG* in pancreatic cancer stem cells and other epigenetic mechanisms, such as histone modification or miRNAs may play a more important direct regulatory role. The latter is supported by two studies. First, Ezh2, the catalytic subunit of Polycomb repressive complex 2 (PRC2) was shown to directly regulate the epigenetic status of the *NANOG* promoter through H3K27me3 (Villasante et al., 2011). Moreover,

miR-134 was first found to directly target *NANOG* in mouse ES cells (Tay et al., 2008). 2) Conversely, because individual CpG dinucleotides found in Fragment 1 of *NANOG* promoter were less methylated in autofluorescent-positive compared to autofluorescent-negative cells, DNA methylation, at the end, could play a role in regulation of *NANOG* gene expression. Along this line, it was shown that methylation of individual CpG dinucleotides could influence the expression of specific genes, such as *MAGEA1* and *IL6* (Nile et al., 2008; Zhang et al., 2004). It should be noted that by manually selecting fragments to be analyzed for our study we may have missed some more important CpG dinucleotides. For example, it was previously shown that the *NANOG* promoter was hypomethylated in CD133-positive-high compared to CD133-positive-low or CD133-negative cells from HCT116 colorectal carcinoma cell line (Wang et al., 2013b). In this study the authors address the methylation status of *NANOG* promoter at position from -1449 to -952, and Fragment 1 in our study covered positions from -1168 to -953. Thus, we could have missed some upstream CpG dinucleotides that could have given more pronounced differences in methylation between autofluorescent-positive and -negative cells. In addition, we only looked at the methylation status of the *NANOG* promoter. It may have been interesting to look at whether the promoters of other pluripotency-associated gens are also differentially methylated between CSCs and non-CSCs. Nonetheless, our bisulfite sequencing data did suggest that CSCs may have a distinct methylation epigenome compared to non-CSCs, and that these differences might be more obvious using genome-wide level analyses.

Unlike bisulfite sequencing analyses, which are more restricted and less globally informative, a genome-wide approach would allow us to discover novel, global and targetable signalling pathways involved in the self-renewal and tumourigenicity of pancreatic CSCs and that are regulated by DNA methylation. Moreover, it is important to mention that the observed mild differences in the methylation of Fragment 1 of the *NANOG* promoter could have been a consequence of epigenetic noise from heterogeneous cell populations. It is known, at least in ES cells, that the levels of *NANOG* expression fluctuate between *NANOG*-high and *NANOG*-low states, which are related, respectively, to ES cell proneness to self-renew or to differentiate (Villasante et al., 2011). Studies done at the single-cell level have revealed substantial gene expression variability even among stem cells (Canham et al., 2010; Chambers et al., 2007; Guo et al., 2010), indicating that CSCs may also display genetic and/or epigenetic heterogeneity in respect to the cell population. Therefore, epigenetic studies would highly benefit from monoclonal

systems, such as single cell-derived tumours and/or clonal in vitro cultures that would minimize the existence of this "noise". Towards this end, Miranda-Lorenzo *et al*., showed that one single autofluorescent-positive cell could generate a tumour *in vivo*, recapitulating the histological heterogeneity of the primary tumour but at the same time providing a system devoid of tumour genetic heterogeneity and multiclonality (Miranda-Lorenzo et al., 2014). Moreover, building upon the principles of CSC-mediated tumour formation and metastasis, where the latter is believed to be a result of a more restricted EMT-like sub-population of CSCs, we reasoned that the CSC heterogeneity should be highest in the primary tumour, less in the metastatic tumour and homogenous in the CSCs isolated from the single cell-derived tumour. Thus we FACS sorted autofluorescent-positive and autofluorescent-negative cells from pancreatic cancer cells of the primary tumour (185), cells from the liver metastasis (A6L), and cells obtained from a tumour generated from one single cell (185scd) (Figure 7A), and performed global methylation analysis, using a 450K Illumina methylation array. Principal component analysis based on normalized β methylation values showed that methylation differences were greater between different tumours (185, A6L and 185scd) than between autofluorescent-positive and -negative cells (Figure 9). Methylation difference between tumours A6L and 185 are most likely due to patient-specific methylation differences, since these tumours originate from different patient, which is in agreement with previous studies (Lokk et al., 2014). It has been shown that the metastatic process is followed by specific changes in DNA methylation profile (Aryee et al., 2013; Reyngold et al., 2014), and since A6L tumour cells are derived from a liver metastasis and 185 tumour cells from the primary tumour, the differences in tumour origin likely accounts for the methylation differences observed, apart from patient-specific changes. Principal component analysis also showed that 185 cells from the single cell-derived tumour were closer to 185 primary tumour from which they were generated and less similar to A6L tumour cells, confirming existence of a tumour-type specific methylation profile. Interestingly, the 185scd tumour cells still fall into a separate group from the 185 primary tumour cells, which could suggest that the methylation profile of 185scd tumour cells represent a methylation signature of a single clone. In line with this hypothesis, DNA methylation analysis studies performed on prostate cancer tumours from geographically distinct primary tumour sites revealed the presence of multiple sub-clonal cell populations that were characterized by distinct DNA methylation profiles (Brocks et al., 2014; Landau et al., 2014). Interestingly, comparing the global methylation levels in autofluorescent-

positive CSCs to autofluorescent-negative cells from each tumour, we observed that autofluorescent-positive cells, as a population, had significantly higher levels of global DNA methylation (Figure 10). This finding is opposite to what was reported before, which we will discuss further below.

We next moved on to look for differently methylated probes. In this regard, we 1) compared autofluorescent-positive and autofluorescent-negative populations of cells in each tumour separately and 2) compared grouped autofluorescent-positive cells from all tumours to grouped autofluorescent-negative cells from all tumours. Since only two biological replicates for autofluorescent-positive and –negative samples from each tumour were available, we used specific criteria, described in the methodology section, to identify hypo- and hypermethylated probes in each pair of autofluorescent-positive and -negative samples. We found that autofluorescent-positive populations exhibited higher numbers of hyper- than hypomethylated probes, 369 vs 77, 1035 vs 343 and 718 vs 127 in primary, metastatic and single cell-derived tumour cells, respectively. In autofluorescent-negative populations, the number of hyper and hypomethylated probes were similar. The higher number of hypermethylated probes observed in the autofluorescent-positive populations could be due to the global increase in methylation levels observed in the autofluorescent-positive population of cells, which suggest that in pancreatic cancer the transition from CSC to non-CSC phenotype is followed by a decrease in global methylation. This observation is in disagreement with a previous study in breast CSCs where the authors showed that CSCs differentiation was followed by an increase in average methylation levels, and these results were similar to the DNA methylation dynamics observed during differentiation of blood and skin stem cells (Bock et al., 2012). Whether pancreatic CSC differentiation has unique methylation dynamics still needs to be confirmed by additional experiments, such as direct quantification of global 5-methylcitosine levels in population of CSCs and non-CSCs; however, the following results investigating the expression level of DNA methyltranspherase between autofluorescent-positive and -negative cells supports this model. Specifically, we discovered that autofluorescent-positive cells express significantly higher levels of DNMT1 and DNMT3a compared to -negative cells (Figure 19), and higher levels of the aforementioned DNA methyltransferases could account for global increase in methylation seen in autofluorescent-positive cells. Jaiswal *et al*, similarly showed a positive correlation between global methylation levels and mRNA expression of DNMTs in patients with impaired spermatogenesis

(Jaiswal D, 2015). An important limitation of our study is the fact that we relied on one marker for the purification and isolation of pancreatic CSCs. While autofluorescence satisfies the requirements of a CSC marker, it is still a relatively new marker and its ability to separate pure CSC populations from non-CSC populations has not been rigorously tested. In fact, Miranda-Lorenzo *et al*, showed that a hierarchy existed within the autofluorescent population, with CD133-positive/autofluorescent-positive cells exhibiting higher tumourigenicity compared to autofluorescent-positive cells (Miranda-Lorenzo et al., 2014). Unfortunately, due to the small percentage of cells expressing both markers, this double positive population would not have yielded enough material for our analyses and therefore we did not entertain its use. Nonetheless, it should be noted that our study would have benefited from parallel analyses performed with additional markers combinations (e.g. CD133-positive vs CD133-negative).

It has been demonstrated previously that pancreatic cancer stem-like cells that display invasive characteristics showed a unique set of genes that were differently methylated compared to non-invasive cells (Sun et al., 2013a). Among these genes were those involved in EMT or genes previously shown to be involved in human embryonic stem cell pluripotency. When we looked for common hyper and hypomethylated probes between tumours (primary vs. metastatic, primary vs. single cell- derived and metastatic vs. single cell-derived) we also found genes involved in processes such as invasion and migration (i.e. *TGFbr3*, *SNAI1* and *VMP1*), stem cell maintenance (i.e. *MSI2*, *CAMK4*), differentiation (i.e. *UACA*, *BDNF* and *HOXD8*) or Notch signalling (*NOTCH4* and *MAML3*). Interestingly, the overlap in differently methylated probes was greater between metastatic and single cell-derived tumour samples. As previously mentioned, we reasoned that cellular heterogeneity would be greater in the primary tumour and would decrease in the metastatic tumour, but would remain relatively homogeneous in the single cell-derived tumour. This reasoning is supported by recent studies in pancreatic and prostate cancer, where it was shown that primary tumours contain many rearrangements, and most of them can also be found in matched metastases. However, the fact that metastases were phylogenetically distant from the primary tumour indicated that one or more driver mutations conferred a selective advantage for metastatic spread (Campbell et al., 2010; Liu et al., 2009). These data show that despite common genomic heterogeneity in primary cancers, most metastatic cancers arise from a single precursor cancer cell. Within the developing metastases, clonal evolution may continue, but probably to a lesser extent than in the primary tumour, making this lesion less polyclonal. Thus, the greater overlap in

differently methylated probes between metastatic tumour and single cell-derived tumour could be because autofluorescent-positive and -negative population of cells in these two tumours are less heterogeneous, resulting in less methylation noise, thus revealing truly important genes for CSC biology. Indeed, we found in this common group of genes ones related to invasion (*MCF2L*, *VMP1*, and SNAI1), stem cell maintenance (*CAMK4*) and Notch signalling (*NOTCH4* and *MAML3*). We next sought to validate and understand if these "hits" were also functionally important for CSCs by quantifying their expression at the mRNA level. It is important to mention that difference in methylation β values were no more than ~10% different between autofluorescent-positive and –negative samples; however, despite this small change in methylation levels we did observed that *MCF2L*, *VMP1*,and *MAML3* genes were at least two fold more expressed in autofluorescent-positive compared to autofluorescent-negative cells.

We paid particular attention to *MAML3*, as this protein is a component of the Notch signalling pathways, which has been shown to be involved in cell proliferation, invasion, metastases, and angiogenesis in a variety of human cancers including pancreatic cancer (Ranganathan et al., 2011). In humans, the three co-activator members *MAML1* (for Mam-like 1; also known as Mam-1), *MAML2* (Mam-3) and *MAML3* (Mam-2) have been identified (Kitagawa et al., 2001; Lin et al., 2002), and studies investigating the functional roles of MAML family members in regulating Notch-based transcriptional events showed that MAML genes collectively are essential in mediating physiological Notch functions (McElhinny et al., 2008). The overexpression of the Notch signalling pathway has been observed in pancreatic cancer (Buchler et al., 2005), and previous studies have also shown that inhibition of Notch-1 using its siRNA or γ-secretase inhibitor (GSI) suppressed cell growth, induced apoptosis and decreased invasion in pancreatic cancer cells (Wang et al., 2006a; Wang et al., 2006b). Moreover, knockdown of Maml2 and Maml3 also led to a reduction in the second-generation sphere-forming capacity of pancreatic cancer cell lines (Brabletz et al., 2011). Taken together, these findings indicated to us that activation of Notch signalling could contribute to CSC self-renewal and aggressiveness. We found that *MAML3* was significantly more expressed in pancreatic CSCs (autofluorescent-positive cells vs autofluorescent-negative cells and sphere-derived cells vs adherent cells; Figure 14), indicating that this gene could play an important role in CSC biology. However, this question still remains to be answered since all attempts to silence this gene by using an established inducible lentiviral-knockdown approach were unsuccessful (Figure 15).

In order to find differently methylated probes with statistical significance, we grouped autofluorescent-positive and compared them to autofluorescent-negative cells from all tumours. Differential methylation analysis was performed using the Limma package (R/Bioconductor software package that provides an integrated solution for analysing gene expression or methylation data (Ritchie et al., 2015)). Of note, to obtain probes that were statistically significant, we had to implement into Limma a "robust method" of analysis. Developed by Phipson *et al*, (Phipson, 2013) this method improves the power to detect differential expression for the majority of genes that are not outliers. Due to higher methylation difference between tumours, than between autofluorescent-positive and -negative populations, we used tumour "origin" as a blocking variable, which allowed us to identify probes most likely to be scientifically relevant to the experimental condition (positives vs. negatives). Using this approach we found, 9,230 and 1,382, hyper or hypomethylated probes, respectively, in autofluorescent-positive populations compared to autofluorescent-negative populations (Figure16). Subsequent IPA (Ingenuity Pathway) analysis of hypermethylated probes revealed pathways related to the Polo-like kinase, DNA damage repair, TGF-ß signalling and epithelial to mesenchymal transition (EMT) (Figure 13). The Polo-like kinase pathway was one of the top pathways represented in our data set, thus making the Polo-like kinases an attractive targets for further studies. A previous study in pancreatic cancer investigated the role of one member of this kinases, Plk-1 and showed it to be highly expressed in pancreatic cancer and its expression was correlated significantly with gemcitabine resistance in human pancreatic adenocarcinoma cells (Gray et al., 2004). However the role of other members, specifically the Plk-3, which appears in our list of genes as hypermethylated in autofluorescent-positive cells, has yet to be determined, though some reports suggest that Plk-3 could function as a tumour-suppressor in pancreatic cancer and that its loss could be important for pancreatic cancer progression (Li, 2012). Thus, it would be interesting to investigate the role Plk-3 in pancreatic cancer (stem) cells, by first confirming its methylation status in autofluorescent-positive vs. autofluorescent-negative cells by bisulfite pyrosequencing and subsequently correlating those results with mRNA expression levels.

There remains much to be done to validate and follow-up on the extensive methylation data generated in the above detailed experiments. While apparent obstacles were encountered throughout the process (e.g. CSC purification using autofluorescence, analysis of methylation data, the need for greater numbers of replicates) our data, in the end, did identify genes, signatures and

pathways unique to CSCs and metastatic CSCs, indicating that in light of experimental difficulties our data clearly show CSC biological relevance. This in encouraging and supports our overall conclusion that CSCs have a hypermethylated genetic signature compared to non-CSCs. Confirmation of this hypermethylation status/profile in pancreatic CSCs using different biological and technical approaches, such as those discussed above, would open the door for the development of drugs that could be used to reverse hypermethylation in this subpopulation of biologically important cells, perhaps restoring the tumour suppressive activities of some genes (e.g. Plk), ultimately effecting the biology and "stemness" of CSCs.

2. TARGETING DNA METHYLATION IN PANCREATIC CANCER STEM CELLS

Although our global methylation array data provided 1) no clear picture of the methylation status of CSCs versus non-CSCs nor 2) gave a list of target genes that we could efficiently validate and show to be necessary for CSC biology, our studies did, however, highlight that CSCs (i.e autofluorescent-positive cells) have a higher level of global methylation compared to their negative counterparts, regardless of the heterogeneity or polyclonality of the CSC populations present in the tumours analysed. This higher global methylation in the autofluorescent-positive population of cells could be explained by differential expression of DNA methyltranspherase genes (Jaiswal D, 2015). While we show that DNMT1 and DNMT3a are significantly highly expressed in autofluorescent-positive cells at the mRNA level, at the protein level we did not see difference between autofluorescent-positive and -negative cells (Figure 19). Using CSCs derived from sphere cultures, we again checked the expression of DNMTs (DNMT1, DNMT3a and DNMT3b) from various primary PDAC cultures, and observed that DNMT1 was the most consistently up-regulated methyltranspherase at both the mRNA and protein level (Figure 20A and 20B). Interestingly, DNMT1 protein expression has been shown to increase with the development of pancreatic cancer, from normal tissue to precancerous lesions (PanINs) to PDAC (Peng et al., 2005). Moreover, DNMT1 has been shown to be essential for the maintenance of hematopoietic stem cells (HSCs)/progenitor cells (Trowbridge et al., 2009), epidermal progenitor cells (Sen et al., 2010) and leukaemia stem cells (Trowbridge et al., 2012). More recently, DNMT1 was shown to be indispensable for mammary stem/progenitor cells (MaSC) and CSC maintenance, and that functional inactivation of this gene drastically reduces mammary tumour formation (Pathania et

al., 2015). The sum of these studies and the observation that pancreatic CSCs express higher level of DNMT1 makes this protein a very attractive and realistic target for chemotherapy. Thus, in order to test the biological role and relevance of DNMT1 and global methylation in the context of pancreatic CSCs, we performed pharmacological targeting-based experiments using the DNA methylation inhibitor Zebularine.

Zebularine is a novel DNA methyltransferase (DNMT) inhibitor and unlike other DNMT inhibitors, it is more stable in aqueous solution and is less toxic *in vitro* and *in vivo* (Valenzuela et al., 2014). Continuous exposure of numerous cancer cell lines to zebularine slowed tumour cell growth as compared to normal human fibroblast cell lines indicating its promise as a chemotherapy agent for cancer treatment. Zebularine also showed a promising anti-cancer effect in pancreatic cancer cells *in vitro* and *in vivo*, leading to apoptosis induction and growth suppression (Neureiter et al., 2007). Even though initial reports showed promise for the use of zebularine in treating pancreatic cancer, no subsequent studies were conducted to further investigate the mechanism of its action nor its specific effect on pancreatic CSCs. This could be due to the negative effects observed in clinical trials using other DNA methyltransferase (DNMT) inhibitors to treat solid tumours, as discussed in the Introduction. Nevertheless, we investigated the effect of zebularine-mediated inhibition of DNMT1 protein in PDAC CSCs, and show that treatment of CSCs with zebularine abrogated their CSC phenotypes. Specifically, we observed 1) a significant decrease in CSC self-renewal, as measured by sphere formation capacity, 2) a reduction of CSC surface marker expression (e.g. CD133) and 3) reduced *in vivo* tumourigenicity in limiting dilution assays (Figure 23, 24 &27). Moreover, the effects were not mediated by induction of apoptotic cell death, nor by strong changes in cell cycle distribution (Figure 25&26). Initial studies in pancreatic cancer cell lines showed that zebularine significantly induced apoptosis and only marginally reduced cell proliferation; however, the concentration of zebularine used in these studies was much higher 1mM in comparison to the 75μM dose used herein. In our hands and using primary PDX-derived cultures, we show that concentrations higher than 100μM were toxic (Figure 21), thus using the concentrations (e.g. 1mM) described by Neureiter *et al.* would surely induce unspecific cytotoxicity. Moreover in the study by Neureiter *et al.*, apoptosis was measured 120 h post treatment, while we assessed apoptotic induction after 72 h following sphere-formation initiation (Neureiter et al., 2007). Possibly the assessment of apoptotic cell death and proliferation in 1st generation spheres at day 7 or even in 2nd generation spheres would have been more informative.

DNMT inhibitors can also mediate their cytotoxic effects through induction of DNA strand breaks (Palii et al., 2008). Indeed, it was shown that zebularine is able to induce DNA damage in leukemic T cell lines and in HCT116 colon cancer cell lines (Ruiz-Magana et al., 2012; Yang et al., 2013b). Activation of DNA damage response can result in two primary outcomes: repair of DNA damage and genomic restoration or, if damaged DNA cannot be sufficiently repaired, execution of cell death or senescence programs. Induction of senescence and/or apoptosis depends on the severity of the stress induced, as has been shown for doxorubicin in MCF7 breast cancer cells (Song et al., 2005) where high doses induced apoptosis due to overwhelming stress while low doses induced senescence as a consequence of less severe damage. Importantly, these outcomes are not necessarily mutually exclusive and they can be subsequently followed in time post treatment exposure. For example it was shown that in human chronic myeloid leukemia (CML) cell lines, treatment with yet another DNA methylation inhibitor, decitabine, induced cellular senescence, and only after prolonged treatment was there activation of mitochondrial-dependent apoptosis (Schnekenburger et al., 2011). Based on this reports, since we did not see induction of apoptosis at early time points (72 h post treatment), the decrease in CSC phenotypes could possibly be explained by initial induction of senescence, followed by induction of apoptosis, which we could have confirmed if spheres had been analysed at later time points (e.g. at day 7 or in 2nd generation spheres). An apparent decrease in the cell cycle distribution in A6L sphere-derived cells treated with zebularine would be in line with our hypothesis regarding initial senescence induction, but histochemical staining for senescence-associated β-galactosidase would need to be performed to definitively confirm this hypothesis. In contrast, 185 sphere-derived CSCs showed no change in cell cycle distribution, which would suggest another mechanism of action apart from senescence and/or apoptosis in zebularine-treated CSCs from this tumour, such as induction of differentiation, for example. In support of this argument, we observed that the expression of the CSC marker CD133 decreased upon zebularine treatment (Figure 23). This decrease was not due to a loss of this population, but rather to an increase in percentage of more differentiated CD133-negative population of cells, suggesting that CD133-positive CSCs were reverting to CD133-negative non-CSCs. Promotion of CSCs differentiation is in line with previous reports showing that *in vivo* treatment of xenografts derived from pancreatic cancer cell lines with zebularine influenced differentiation patterns, such as CK7 up-regulation and down-regulation of dedifferentiation markers, such as c-kit and Notch (Neureiter et al., 2007). We did not measure such changes in our

zebularine treated tumour, and as such more direct analyses are still needed to confirm this alternate working hypothesis, such as immunohistochemistry for differentiation markers (-i.e. CK19) in tumours derived from zebularine treated sphere cells. Moreover, since there is a general assumption that CSCs are more resistant to chemotherapy and/or radiotherapy, it would be interesting to see if zebularine-mediated differentiation of CD133-positive CSCs to CD133-negative cells is followed by acquired sensitivity to conventional chemotherapy, such as gemcitabine. All together our results suggest that zebularine can influence CSC behaviour, *in vitro* and *in vivo*, but the precise mechanism of action is still unclear.

Over expression of DNMT1 in pancreatic CSCs and loss of CSC phenotypes following DNMT1 down-regulation by zebularine would suggest that PDAC CSCs are specifically dependent on this protein. In this line, it would be interesting, to perform shRNA mediated knock-down of DNMT1 to directly support this hypothesis. However, a more likely explanation is that since zebularine is a global demethylation drug and its ability to alter CSC phenotypes is likely linked to the reactivation of very diverse set of genes that could influence CSC properties. After confirming that zebularine indeed induces global demethylation in sphere-derived CSCs (Figure 29), we moved forward to identify target genes potentially reactivated by zebularine. We examined the global-methylation profile of zebularine-treated CSCs, using the previously mentioned 450K Illumina array methodology, specifically comparing sphere-derived untreated CSCs with sphere-derived zebularine-treated CSCs. Among the many genes that were demethylated, we focused our attention on miRNAs because epigenetic alterations in cancer are often times a result of miRNA deregulation (Saito and Jones, 2006). For example, miR-145 is a well-known tumour-suppressor miRNA that is down-regulated in many human cancers owing to aberrant DNA methylation of its promoter (Suh et al., 2011). Hypermethylation of the miR-9 family has also been reported in hematopoietic malignancies (Roman-Gomez et al., 2009) and renal cell carcinoma (Hildebrandt et al., 2010). A more recent study by Cioffi *et al*, highlighted the role of miRNAs, specifically the miR-17-92 cluster in pancreatic cancer stem cell quiescence and chemoresistance (Cioffi et al., 2015). Specifically, the authors found the miR-17-92 cluster, composed of six members (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a), to be markedly suppressed in pancreatic CSCs as compared with their more differentiated and chemosensitive counterparts. Transcriptional regulation of this cluster can be regulated by various transcription factors such as c-MYC (Barski et al., 2009), E2F1 or E2F3 (Sylvestre et al., 2007). Moreover, it was recently shown that the miR-

17-92 cluster can be regulated by DNA methylation and this contributes to the pathogenesis of pulmonary fibrosis. Specifically, treatment with 5′-aza-2′-deoxycytidine in a murine bleomycin-induced pulmonary fibrosis model enhanced miR-17-92 cluster expression and attenuated pulmonary fibrosis (Dakhlallah et al., 2013).

Encouraged by this study, we looked at our methylation array data from sphere-derived untreated CSCs and zebularine-treated CSCs and found that zebularine treatment induced hypomethylation of CpG dinucleotides found adjacent to miR-17-92 locus (Figure 31). This hypomethylation effect was followed by an increase in the expression of the miR-17-92 members, with notable increases in miR-19a and -19b (Figure 32) across independent sets of samples. In addition, increase in miR-17-92 members was followed by down-regulation of predicted target genes, such as p21, TGFBR2, ALK4, SMAD2 and SMAD4 (Figure 33), all of which have been shown to be necessary and/or required for pancreatic CSCs (Cioffi et al., 2015; Lonardo et al., 2011). These results were in line with data shown by Cioffi *et al,* where lentiviral-mediated overexpression of miR-17-92 family members similarly decreased the mRNA expression level of the aforementioned target genes (Cioffi et al., 2015). Moreover, this gain-of-function experiments showed that overexpression of miR-17-92 in the CSC compartment was able to push CSCs towards a more differentiated state, decreasing their self-renewal capacity and *in vivo* tumourigenicity-phenotypes similar to what we observed with zebularine treatment alone. The mechanism behind the decrease expression of miR-17-92 cluster in pancreatic CSC is not well defend nor did Cioffi et al. propose how this cluster is differentially regulated in CSCs, however, our observation that zebularine-mediated DNA hypomethylation effects this cluster suggest that DNA methylation could be responsible for silencing this cluster in PDAC CSCs. Thus zebularine's effect on the CSC compartment (e.g. decrease in sphere formation and *in vivo* tumourigenicity), could be explained by reactivation of the miR-17-92 cluster. Indeed, while antagomiR inhibition of the miR-17-92 cluster in more differentiated non-CSCs reprogrammed these cells towards a CSC-like phenotype, the effect could be partially reversed by zebularine treatment (Figure 34), supporting our underlying hypothesis.

Interestingly, we also observed that zebularine treatment induced a pronounced decrease in p21 protein levels. Other studies describing the effects of DNA methylation inhibitors on cancer cells have shown that demethylation increased p21 expression level and modulated cell cycle progression (Nakamura et al., 2013; Yang et al., 2013b). Furthermore, Zhu *et al*. reported that

induction of p21 was p53 dependent (Zhu et al., 2004). Studies in colorectal cancer showed that zebularine induction of p21 is only observed in cells that are p53 proficient (Yang et al., 2013b). Since our primary PDAC cells are p53-deficient, the difference in p53 status could account for differences between our results and previous findings. The majority of zebularine-related studies investigated the effects of demethylation on bulk tumour cells. Thus silencing of p21 could be a response specific to pancreatic CSCs, related to dysregulation of miRNAs from the miR-17-92 cluster targeting this gene. Cioffi *et al*, showed that p21 is consistently up-regulated in chemoresistant CSCs (Cioffi et al., 2015). They also showed that shRNA-mediated down-regulation of p21 resulted in impaired self-renewal and chemoresistance. Taken together these results support the model that effects of zebularine are mediated through the reactivation of the miR-17-92 cluster, which in turn down-regulates target genes, such as p21, which in CSCs is extremely important for preventing pancreatic CSC exhaustion. Additional experiments are still needed to dissect the apparent connection between the miR-17-92 cluster and hypermethylation in CSCs, such as in-depth dissection of the methylation status of the miR-17-92 cluster and their target genes in CSCs and non-CSCs using bisulfite pyrosequencing, as well definitely determining the degree of demethylation that occurs at this cluster upon treatment with de-methylation drugs, such as zebularine.

3. ROLE OF DNA DEMETHYLASES IN PDAC CSCs

Taken together, our previous findings strongly highlighted the importance of DNA methylation and DNMT proteins, specifically DNMT1 methyltranspherase, in pancreatic CSCs. In line with this conclusion, we were pleasantly surprised to discover by RNA-seq analysis that genes working in an opposing manner to DNA methyltranspherase were up-regulated in spheres versus adherent cells. TET genes, composed of three members TET1, TET2 and TET3, are known key players in cytosine demethylation and in the control of cellular differentiation and transformation (Tahiliani et al., 2009). Expressions of TET proteins are tightly regulated at the transcriptional level. For example, in mouse embryonic stem cells (mESCs) both TET1 and TET2 are positively regulated by OCT4, and their mRNA levels decrease dramatically upon mESC differentiation. In contrast, TET3 is significantly up-regulated during differentiation (Delhommeau et al., 2009). We found that TET1 and TET2 were significantly overexpressed at

the mRNA level in CSCs when compared to more differentiated cancer cells (Figure 35), however, when we checked at the protein level, we found that Tet2 was strongly down-regulated in CSCs (Figure 36). Decreased expression of Tet2 protein has been reported in many blood tumours (Delhommeau et al., 2009) and in a number of solid cancers, such as liver, breast and melanoma (Yang et al., 2013a). These findings in combination with our observations that the Tet2 protein is down-regulated in pancreatic CSCs, and in light of the fact that the role of TET2 in pancreatic cancer and specifically in pancreatic cancer stem cells has not been investigated before, promoted us to investigate the role of this protein in pancreatic CSCs biology and tumourigenesis.

In loss-of-function experiments using a lentiviral approach, we silenced TET2 in more differentiated non-CSCs that expressed high levels of Tet2 protein assuming that such an approach would push these cells toward a more CSC state. Indeed, we observed a significant increase in the sphere and colony formation capacity (Figure 39) and *in vivo* tumourigenicity of Tet2 silenced non-CSCs (Figure 42). Similarly to our findings, loss of TET2 in leukaemia resulted in an increase in the haematopoietic stem cell compartment (Quivoron et al., 2011). We did not observe, however, changes in expression of pluripotency genes upon Tet2 knock-down (Figure 38) and studies in ESC showed that deficiency in TET1, TET2 or TET1/2 in ESCs does not seem to affect ESC maintenance or pluripotency (Williams et al., 2011). Thus we hypothesize that loss of TET2 in more differentiated cells, could endow them with more aggressive and proliferative phenotype. *In vitro* migration assays indicated that these cells had higher proliferation rates. More direct proof is still needed to determine how TET2 loss promotes a CSC phenotype. For example, it would be interesting to analyse the cell cycle profile of shTet2 cells to determine whether TET2 silencing increases proliferation. Moreover, it would be interesting to see whether in *in vivo* serial transplantation experiments, shTet2 cells maintain tumour growth, indicating an endowment with CSC phenotypes or whether these cells eventually exhausted due to extensive proliferation. It is likely that by assessing pieces of the more global methylation puzzle we are only observing snapshots of the bigger picture. Perhaps combining Tet2 loss with demethylation or miR-17-92 antagimiRs would result in a more concrete phenotype.

Lastly, decreased Tet2 protein levels in CSCs in light of its high mRNA expression is intriguing. Published data describing the mechanisms controlling Tet protein levels are diverse and abundant. Recently, it was found that Tet2 protein levels can be regulated by IDAX (Ko et al., 2013), which is often mutated or overexpressed in cancer. Our preliminary data show that IDAX

is overexpressed in CSCs, suggesting that its higher level in CSCs could promote degradation of the Tet2 protein. Finally, levels of Tet proteins can be modulated by more than 30 miRNAs. Overexpression of miR-22 in the haematopoietic or mammary gland compartment was shown to inhibit TET-mediated hydroxymethylation and promotes leukaemogenesis or breast cancer, respectively (Song et al., 2013a). Indeed, our preliminary findings show that various miRNA targeting Tet2, are more expressed in spheres versus adherent cells (Figure 43).

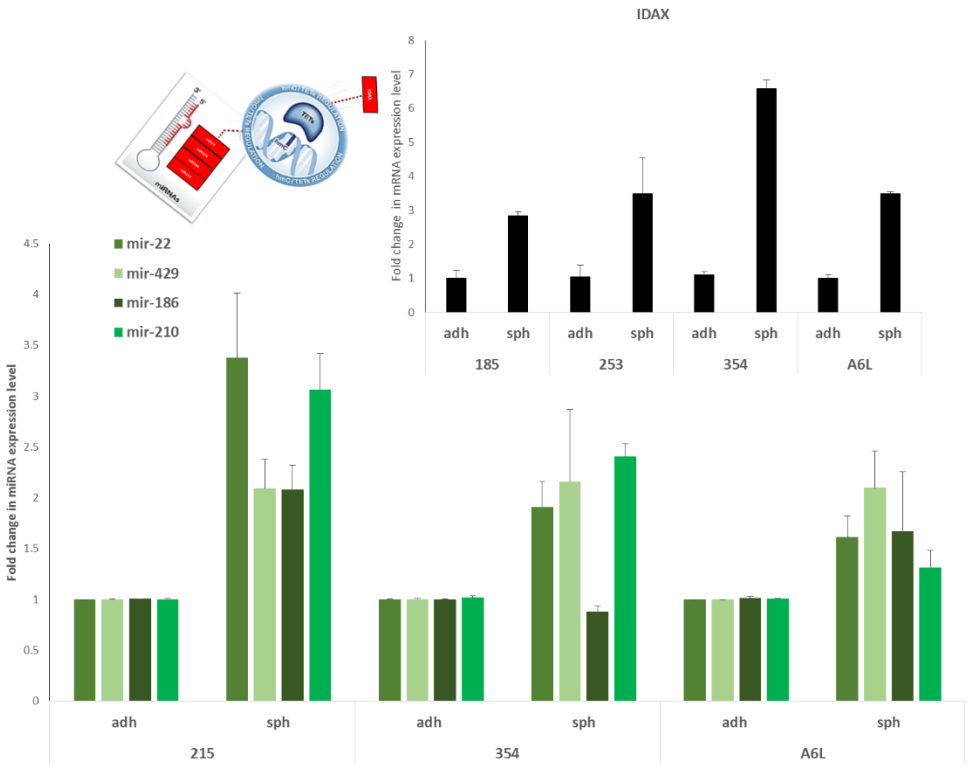

Figure 43. Possible mechanism regulating Tet2 protein stability. IDAX was shown to promote Tet2 protein degradation, and RTqPCR analysis on a panel of primary PDAC adherent and sphere cultures show that IDAX is more expressed in CSCs (spheres) versus non-CSCs (adherent) (right panel). Moreover, Tet2 can be regulated by numerous miRNAs. We show here that miR-22, -429,-186 and -210 are more expressed in spheres versus adherent cells. Data were normalized to β-actin or snord44 and are represented as fold change compared to adherent cells.

Taken together, our data highlight that pancreatic CSCs have a unique and interesting pattern of expression of DNA methylation and de-methylation enzymes, suggesting that these two opposite processes could influence the delicate process that govern CSCs plasticity. However, many questions still remain unanswered, such as 1) are these two proteins connected, or do they operate in independent fashion? 2) Would decrease in DNMT1 in CSCs induce TET2 expression? 3) Is

the CSC state characterized by loss of 5hmC level? 4) Is epigenetic regulation of miR-17-92 cluster by DNMT1 and TET2, tip of the scale in modulating key pathways for pancreatic CSCs biology, such as Nodal/Activin/Tgf-β or p21?

It is likely that inter-conversion between CSCs and differentiated cells could be mediated by slight changes in the expression of DNMT1 and TET2 proteins, as depicted in the model described on Figure 44. As with most cellular processes, these two opposing proteins likely represent the ying and yang of a seesaw methylation cascade that governs CSC "stemness" in combination with numerous other factors including the miR-19-92 cluster. Deciphering how we can manipulate this balance to favour CSC differentiation would significantly facilitate our ability to eliminate these cells and possible cure this devastating disease.

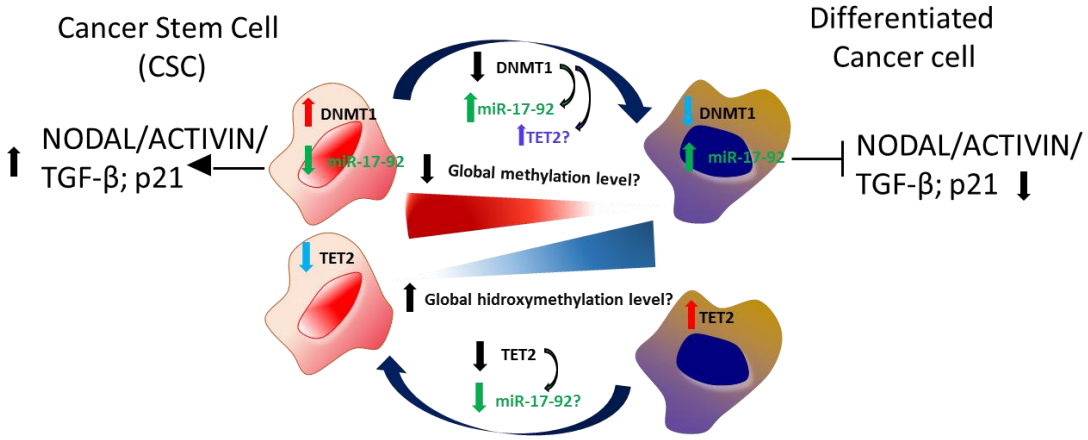

Figure 44. Proposed model of DNMT1 and TET2 regulation of CSC epigenetic plasticity.

CONCLUSIONS

We have accumulated compelling evidence indicating that DNA methylation and de-methylation play important roles in the biology of pancreatic cancer stem cells. In summary we found that:

1. DNA methylation could be responsible for the differential expression of the pluripotency-associated gene *NANOG* in autofluorescent CSCs versus non-CSCs, but before a definitive conclusion can be made it would be necessary to 1) dissect every CpG dinucleotide in the *NANOG* promoter and 2) perform additional analyses in monoclonal populations of cells or at the single cell level.
2. Genome-wide approaches to study DNA methylation in CSCs requires sufficient number of sample replicates that are not highly heterogeneous in order to obtain clear differences in gene methylation-levels that are functionally relevant for CSC biology.
3. Pancreatic CSCs show higher global methylation levels and express higher levels of the DNA methyltranspherase DNMT1.
4. Inhibition of the DNMT1 protein using the DNMT inhibitor zebularine decreases global methylation and decreases CSCs phenotypes such as: reduced expression of pluripotency-associated genes, reduced expression of the CSC cell surface marker CD133, diminished sphere formation capacity and reduced *in vivo* tumourigenicity.
5. Zebularine inhibitory effects on pancreatic CSCs are mediated through reactivation of miRNAs pertaining to the miR-17-92 cluster (normally down-regulated in this population of cells), as antagomir-mediated inhibition of the miR-17-92 in differentiated non-CSCs promoted a CSC phenotype in these cells, but could be reversed by zebularine treatment.
6. Reactivation of miR-17-92 cluster by zebularine treatment down-regulated known miR-17-92 target genes, such as members of Nodal/Activin/TGFβ signalling pathway (Alk4, Smad2, Smad4) as well as regulators of cell cycle (p21).
7. The DNA demethylase TET2 is overexpressed at the mRNA level in pancreatic CSCs compared to non-CSC cells, but its protein levels are decreased in CSC. Several negative regulators of the TET2 protein are more expressed in CSCs, such as IDAX or the miRNAs miR-22, -429,-186 and -210.
8. TET2 is down-regulated at the protein level in pancreatic CSCs compared to non-CSC cells, and shRNA-mediated knock-down of TET2 in more differentiated non-CSC cells

confers increased "stemness" as measured by enhanced sphere-formation and colony formation capacity and *in vivo* tumourigenicity.

9. Pancreatic CSCs and non-CSCs display interesting expression patterns of proteins involved in the regulation of the DNA methylation landscape, and subsequent modification of these players balances CSC plasticity.

CONCLUSIONES

Hemos acumulado pruebas convincentes que indican que la metilación y de-metilación del ADN desempeñan papeles importantes en la biología de las células madre de cáncer de páncreas. En resumen, se encontró que:

1. Metilación del ADN podría ser responsable de la expresión diferencial del gen asociado a pluripotencia, NANOG en autofluorescentes CSCs versus no-CSC, pero antes de hacer una conclusión definitiva es necesario 1) diseccionar cada dinucleótido CpG en el promotor del NANOG y 2) realizar análisis adicionales en poblaciones monoclonales de células o en el nivel de células individuales.
2. Uso de un análisis de metilación de alto rendimiento para el estudio de la metilación del ADN en células madre cancerosas requiere suficiente número de réplicas de la muestra que no son muy heterogéneo con el fin de obtener claras diferencias en el niveles de metilación de genes que son funcionalmente relevante para la biología de CSCs.
3. Las CSCs pancreáticas tienen un nivel de metilación global más alto que las células diferenciadas y expresan mayores niveles de la methyltranspherase DNMT1.
4. La inhibición de la proteína DNMT1 usando el inhibidor de DNMTs zebularine disminuye la metilación global y disminuye fenotipos CSC tales como: reducción de la expresión de genes de pluripotencia, reducción de la expresión del CSC marcador de superficie celular CD133, la disminución de capacidad de formación de esfera y reducion en tumorigenicidad *in vivo*.
5. Efectos inhibitorios del zebularine sobre células madre cancerosas pancreáticas están mediados por la reactivación de miRNAs pertenecientes al clúster miR-17-92 (normalmente menos expresado en esta población de células), porque la inhibición de la miR-17-92 mediada por antagomir en la celulas no diferenciada non-CSCs promovió un CSC fenotipo en estas células, pero podría ser revertida por tratamiento con zebularine.
6. Reactivación de miR-17-92 clúster con tratamiento de zebularine disminuye los conocidos genes diana del miR-17-92 clúster, como los miembros de Nodal /Activina /TGFβ (Alk4, Smad2, Smad4), así como reguladores del ciclo célular (p21).
7. El ADN demetilasa gen TET2 está sobre expresado en el nivel de mRNA en células madre cancerosas pancreáticas en comparación con las células más diferenciadas non-CSC, pero

su nivel de proteína esta reduzido en CSC. Varios reguladores negativos de la proteína Tet2 están más expresados en células madre cancerosas, como IDAX o miRNAs miR-22, -429, -186 y -210.

8. TET2 está menos expresado en el nivel de proteínas en células madre cancerosas de páncreas en comparación con las células más diferenciadas, non-CSC y shRNA ‘’knock-down’’ de TET2 en las células más diferenciadas confiere incrementó "troncalidad", medido por una mayor formación de esfera, la capacidad de formación de colonias y una mayor tumorigenicidad *in vivo*.
9. Las células madre cancerosas de páncreas CSCs y non-CSCs muestran un interesante patrón de expresión de las proteínas implicadas en la regulación del paisaje de metilación y la posterior modificación de estos jugadores equilibra plasticidad de las CSCs.

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